การตรวจพบเชื้อ นีโอสปอร่า แคไนนุ่ม (*Neospora caninum*) และผลกระทบต่อสมรรถนะของระบบสืบพันธุ์ในฝูงโคนมไทย

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IDENTIFICATION OF *Neospora caninum* AND ITS IMPACT ON REPRODUCTIVE PERFORMANCE IN THAI DAIRY HERDS

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A Dissertation Submitted in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy Program in Theriogenology

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การศึกษานี้มีวัตถุประสงค์เพื่อ (1) ตรวจวินิจฉัยและยืนยันเชื้อโปรโตซัว *N. caninum* (NC) ในเนื้อเยื่อลูกโคแท้ง พร้อมทั้งกำหนดแนวทางวิธีการตรวจแยกเชื้อ และ (2) เพื่อสำรวจวิจัยผลกระทบของ NC ต่อสมรรถนะของระบบสืบพันธุ์ และประเมินความเสี่ยงการแท้งลูกของแม่โคนมในฝูงที่ติดเชื้อนี้ ตรวจพบ ทาชิซ้อยท์ ของเชื้อ NC โดยวิธี อิมมูโนฮิสโตเคมี ในสมองลูกโค 1 ตัวจากจำนวนลูกโคแท้ง 22 ตัว ลูกโคที่ตรวจพบ NC นี้แท้งจาก แม่โคที่ตรวจพบภูมิคุ้มต่อโรคนี้ ด้วยวิธี ไอเอฟเอ ส่วนแม่โค 21 ตัว และลูกตรวจไม่พบภูมิคุ้มต่อโรค การกำหนดแนวทางวิธีตรวจแยกเชื้อศึกษาจากแม่โค 4 ตัวที่พบภูมิคุ้มต่อโรค เนื้อเยื่อสมองของแม่โคและลูกหลังคลอด ใช้สำหรับการแยกเชื้อโดยเพาะเชื้อโดยตรงในเซลล์เพาะเลี้ยง วีโร และ วิธีฉีดเข้าหนูแฮมสเตอร์ *(Mesocricetus auratus)* หนู 4 กลุ่มทดลอง (ฉีด4 ตัว ควบคุม 2 ตัว) ได้รับการฉีดเนื้อเยื่อสมองบดจากแม่โคและลูก 2 ตัว แม่โค ลูกโคและหนูทั้งหมดไม่พบอาการผิดปกติในระหว่างการศึกษาจนถึงวันฆ่าเพื่อ เก็บเนื้อเยื่อส่วนต่างๆจากแม่โค ลูกโค และหนูไปศึกษาต่อโดยวิธีย้อมสี HE, IHC และตรวจ PCR ตัวอย่างเลือดแม่โคและลูกโคหลังคลอดก่อนกินนมน้ำเหลือง 3 คู่ตรวจไม่พบภูมิคุ้มต่อโรค แต่พบแม่โคและลูก 1 ตัวมีภูมิคุ้มต่อโรค (IFA) ที่ระดับ 1:100 และ อีไลซ่าที่ 69.3% และ 73.1% ตามลำดับ ผลการย้อมสี IHC ตรวจพบเชื้อปาราสิต ซิสต์เฉพาะในสมองลูกโคที่ตรวจพบภูมิคุ้มโรคแรกคลอด แต่ไม่พบในเนื้อเยื่ออื่นจากแม่โค ลูกโคและหนูแฮมสเตอร์ การยืนยันโดยวิธี PCR ในการตรวจดีเอ็นเอ ของนีโอสปอร่าไม่พบในสมองแม่โค แต่พบในสมองลูกโคที่ตรวจพบซิสต์ และสมองของหนูแฮมสเตอร์ 2 ใน 4 ตัวที่ได้รับการฉีดสมองจากลูกโคตัวนี้ การศึกษานี้พบว่าดีเอ็นเอ NCมีความเหมือน 99% กับส่วน 225bp ของส่วนอ้างอิง NC1 และให้ชื่อใหม่ว่า Thai-B1 ไม่สามารถแยกเชื้อ NC โดยวิธีเพาะเชื้อโดยตรง การศึกษานี้สรุปได้ว่าอุบัติการติดเชื้อจากแม่ที่มีภูมิต่อ NC สู่ลูกในขณะอุ้มท้อง ต่ำกว่าในที่มีรายงานมาก่อน จากผลการศึกษาทางซีรั่มวิทยา IHC และ PCR นอกจากนี้แม่โคที่เคยตรวจพบภูมิคุ้มต่อโรค NC อาจให้ผลลบเมื่อตรวจก่อนคลอด มีผลทำให้ผลการตรวจภูมิคุ้มในลูกต่ำกว่ารายงานอื่น ผลการศึกษาพบว่าหนูแฮมสเตอร์ *(M. auratus)* อาจไม่ไวต่อการติดเชื้อต่อสเตรน Thai-B1 และวิธีการแยกเชื้อวิธีนี้ควรมีการศึกษาต่อไป รายงานนี้เป็นรายงานแรกที่พบการติดเชื้อจากแม่สู่ที่มีการติดเชื้อตามธรรมชาติลูกขณะอุ้มท้องในประเทศไทย

การศึกษาผลกระทบต่อสมรรถนะการสืบพันธุ์ในฟาร์มโคนมที่ตรวจพบแม่โคมีภูมิคุ้มต่อ NC จำนวน 12 ฟาร์ม จำนวน 216 ตัว ที่มีโคนมพันธุ์ผสมโฮลสไตน์ 4-29 ตัวต่อฟาร์ม ในจังหวัดนครปฐมระหว่างเดือน มกราคม ถึง ตุลาคม พ.ศ. 2547 เจาะเลือดแม่โคทุกตัวเดือนละครั้งติดต่อกันนาน 10 เดือน พร้อมเก็บบันทึกข้อมูลผสมเทียม การคลอด การแท้งลูก สถานภาพการติดเชื้อพบ12.9% (พิสัย 5.9-30%) ผลการวิเคราะห์ข้อมูลไม่พบความแตกต่างทางสมรรถนะการสืบพันธุ์ระหว่างแม่โคติดเชื้อ และไม่ติดเชื้อ (P>0.05) นอกจากนี้พบความแตกต่างอย่างมีนัยสำคัญทางสถิติ เมื่อเปรียบเทียบสมรรถนะการสืบพันธุ์ระหว่างฟาร์มที่ศึกษา (P=0.0001 และ P=0.0005) ไม่พบความเสี่ยงต่อการแท้งลูกในแม่โคที่มีภูมิคุ้มโรค (RR=2.25; 95%CI=0.64-7.9) สรุปได้ว่าการติดโรค NC มามีผลต่อสมรรถนะของการสืบพันธุ์ของโค การที่จะปรับให้สมรรถนะระบบสืบพันธุ์ที่ต่ำลงในฝูงโคนมให้ดีขึ้นนั้น ควรดูการจัดการฟาร์มและปัจจัยอื่นๆที่เกี่ยวข้อง

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THAN KYAW: IDENTIFICATION OF *Neospora caninum* AND ITS IMPACT ON REPRODUCTIVE PERFORMANCE IN THAI DAIRY HERDS. THESIS ADVISOR: ASSOC. PROF. CHAINARONG LOHACHIT, PhD, THESIS CO-ADVISORS: ASSOC. PROF. PRACHIN VIRAKUL, PhD, WANTANEE KALPRAVIDH, PhD, pp.59
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The aims of this study were (1) to identify *N. caninum* (NC) in the aborted fetuses and to establish an isolation guideline and (2) to investigate the impact of NC on the reproductive performance and abortion risk in dairy cows of NC-seropositive herds. NC tachyzoites were detected, by IHC, in the brain of one of 22 fetuses and its dam was NC seropositive by IFA. The other fetuses and their dams were seronegative. For isolation guideline, the brains of 4 seropositive pregnant cows and their calves, after parturition, were used for direct culture in vero cells and bioassay in hamsters (*Mesocricetus auratus*). Four groups (4 treated and 2 controls each) of hamsters were treated with the brain homogenates of 2 cows and their calves. All cows, calves and hamsters showed no clinical illness until they were euthanized. Selected tissues of cows, calves and hamsters were tested by HE, IHC and PCR. At calving, sera of 3 cows and precolostral blood of their calves were seronegative. The sera of one cow and its calf were seropositive (IFA titer of 1:100 each and ELISA value of 69.3% and 73.1% respectively). A tissue cyst was detected in the brain of this calf by IHC and *Neospora* DNA was also PCR-amplified but not from the brain of its dam. The parasites were not detected in the other tissues of all cows, calves and hamsters. Parasite DNA was also amplified from brains of two of four hamsters treated with the brain homogenates of IHC positive calf. The DNA was not amplified from the other dam-calf pairs and hamsters. Partial sequencing of the NC DNA showed the 99% identities of 225 bp with the reference sequence of NC1 strain. It was designated as Thai-B1. Parasite isolation was not successful. It was concluded that vertical transmission may be much lower than previous reports, as evidenced by serology, IHC and PCR. Also chronically infected cows may have seronegative conversion and the antibody titers of their calves at parturition may be much lower than the other reports. The hamsters (*M. auratus*) may not be susceptible enough to *N. caninum*. Whether to use the isolation method in this guideline needs further clarification. This is the first report of *Neospora*-infection in aborted fetus and congenitally infected calf in Thailand.

Holstein Friesian crossbred cows (total: 216) from 12 NC-seropositive farms in Nakhon Pathom were used to study the impact of *Neospora* infection on the reproductive performance. Blood samples were collected monthly for 10 months. The records of AI, calving and abortions were obtained from the Ratchaburi AI center and farm records. Herd seroprevalence was 12.9% (range 4.76 to 30%). Reproductive performances in seropositive and seronegative cows were the same (P>0.05). But the performances were considerably lower then the other reports. Highly significant differences in the reproductive performance among herds were found (P=0.0001 to 0.0005). The risk of abortion was also not associated with the *Neospora* seropositivity of the cows (RR=2.25; 95%CI= 0.64 to 7.9). It was concluded that in herds with relatively low seroprevalence, *Neospora* seropositivity had no impact on the reproductive performances of the dairy cows. The inferior performance of the cows and large variation among herds indicate the need for the check of management and/or other factors for the improvement.

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Field of study: Theriogenology
Academic year: 2005

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ABBREVIATIONS

ABC	Avidin-biotin complex
AI	Artificial insemination
bp	base pairs
BRSV	Bovine respiratory syncytial virus
BVD	bovine diarrhoea virus disease
cELISA	Competitive enzyme-linked immunosorbent assay
CI	Confidence interval
DAB	3,3'-diaminobenzidine tetrahydrochloride
DAT	Direct agglutination test
DLD	Department of Livestock Department
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide-triphosphate
DW	Distilled water
EDTA	Ethylene-diamine-tetra-acetate
HE	Hematoxylin and Eosin
IBR	Infectious bovine rhinotrachitis
IFAT	Indirect fluorescent antibody test
IgG	Immunoglobulin
IHC	Immunohistochemistry
IU	International unit
mM	Milimolar
NC1	*Neospora caninum* strain 1
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3	Parainfluenza 3
pM	Pica mole
RPMI	Roswell Park Memorial Institute.
SD	Standard deviation
SE	Standard error
UV	Ultra-violet

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Neosporosis, after two decades of its finding as an unidentified organism and continuous works by researchers, is now well recognized as an important abortifacient protozoan disease in cattle industry worldwide (Bjerkås *et al*., 1984; Dubey and Lindsay, 1996; Anderson *et al*., 2000; Dubey, 2003). This parasite also infects various domestic and wild animals such as buffaloes, horse, goats, sheep, deer, camels, dog, cat, birds, foxes, and coyotes (Dubey and Lindsay, 1996; Dubey, 1999a). The studies show that the neosporosis is more prevalent in cattle, buffaloes and canids. The *Neospora* seroprevalence may be as high as 87% in dairies (Wouda *et al*., 1999a), 24% in beef (Sanderson *et al*., 2000), 68% in buffaloes (Dubey *et al*., 1998) and 31.3% in farm dogs (Sawada *et al*., 1998). The life cycle and epidemiology of this parasite is not fully known. At present, dogs and coyotes are the only known definitive hosts for the *Neospora* parasite (McAllister, *et al*., 1998a; Gondim *et al*., 2004a). Dogs are important animals for transmission of this disease among cattle farms. The relationship of *Neospora* transmission between cattle and farm dogs has been reported elsewhere (Wouda *et al*., 1999b; de Souza *et al*., 2002).

The estimated economic loss per year due to neosporosis was at A$ 85 million in dairy and A$ 25 million in beef cattle industry in Australia and NZ$ 17.8 million in the dairy industry in New Zealand (Reichel, 2000). In California dairy industry, it was estimated that about US$ 35 million per year was lost due to abortions by neosporosis (Dubey, 1999b). Until now, the lack of efficient vaccines and treatment for the neosporosis is even more alarming and threatening the economy of the cattle industry.

It is assumed that *Neospora* is not infectious to human as antibodies are not detected in women with repeated abortions (Petersen *et al*., 1999) and agricultural workers (Graham *et al*., 1999). Only 6.7% seropositivity was reported from the result of 1,029 serum samples in California where major cause of abortions in dairy cattle was recognized due to *Neospora* (Tranas *et al*., 1999).

1. 2 Literature review

1.2.1 *History* and *Life Cycle of N. caninum*

The *Neospora* parasite was first reported as an unidentified cyst-forming protozoan causing menigoencephalitis and myositis in dogs (Bjerkås *et al*., 1984). It was morphologically similar to *Toxoplama gondii* but did not react serologically with *T. gondii*. The parasite was first isolated from the congenitally infected puppies (Dubey *et al*., 1988a) and named as *Neospora caninum* (family *Sarcocystidae*, phylum *Apicomplexa*) by Dubey and his colleagues in 1988 (Dubey *et al.,* 1988b). Ten years later, a new serologically distinct species of *Neospora*, *N. hughesi*, was isolated from an adult horse (Marsh *et al*., 1998) but its presence and relationship to cattle and other animals has not been known yet. The parasite can be observed mainly as tachyzoites, tissue cysts and oocysts. Tachyzoites are ovoid, lunate or globular and measure about 3 to 7 × 1 to 5 μm depending on the stage of development and can be found in cells of various tissues of the body while tissue cysts, mostly found in the central nervous system and peripheral nerves, are oval and as large as 107 μm long with a cyst wall thickness of up to 4 μm (Dubey *et al*., 1988b; Dubey and Lindsay, 1996; Speer *et al.*, 1999). More details of morphological, ultrastructural, serological and molecular distinctions of *N. caninum* from other coccidia have recently been reviewed by Dubey *et al.* (2002).

Although the complete life cycle is not fully known, it basically comprises asexual and sexual part of life cycle. The pathway of *Neospora* transmission under current knowledge is shown in Fig 1. Dogs were experimentally (McAllister *et* al., 1998a; Lindsay *et al.*, 1999; Dijkstra *et al.,* 2001) and naturally (Basso *et al*., 2001) proved as definitive hosts. Recently, coyotes are reported as another definitive host (Gondim *et al*., 2004a) which indicates the important path of transmission in and from the world life to the domesticated animals. Dogs eat infected tissues from the intermediate hosts, shed oocysts in the feces and contaminate feeds and water of the intermediate hosts such as cattle. Dijkstra *et al.,* (2001) demonstrated that dogs shed oocysts after ingestion of bovine placenta infected with *N. caninum*. In contrast to dogs, red foxes (*Vulpes vulpes*) did not shed oocysts after feeding infected intermediate host tissues (Schares *et al.,* 2002). The oocysts containing sporozoites are sexually produced and excreted by the infected dogs. These oocysts are sporulated within 3 days after shedding and ready to infect to the susceptible animals (Dubey, 1999b). Tachyzoites are released and distributed into the various tissues of the hosts. The dogs can also be an intermediate host as well. Cats and Mustela species are not definitive hosts (McAllister *et al.,* 1998b; McAllister *et al.,* 1999).

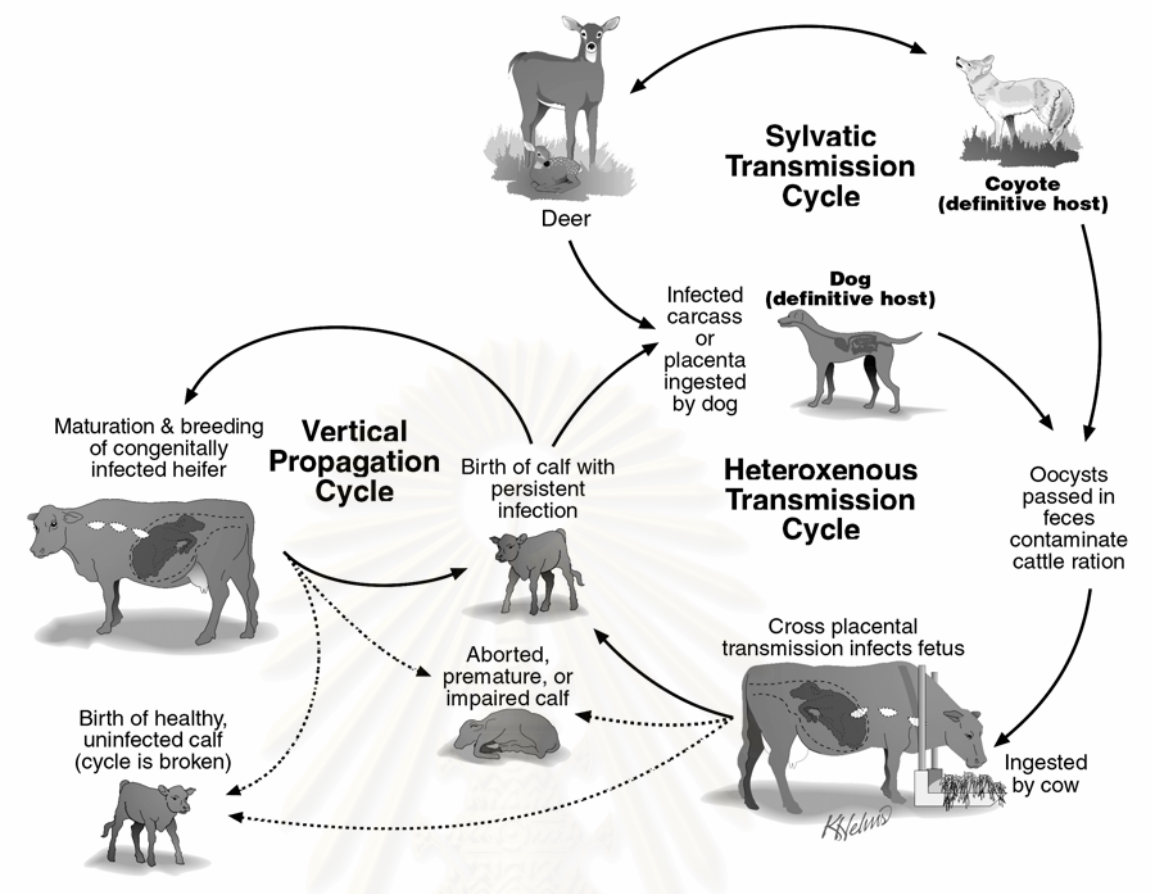

Fig. 1. New *Neospora caninum* life cycle under current knowledge; previous sylvatic hypothesis has been proved. (Kindly provided by M.M. McAllister and Kerry Helms, University of Illinois, USA).

1.2.2 *Disease Transmission*

Two types of *Neospora* transmission are recognized: (1) Vertical propagation or transplacental transmission in which the parasite is passed from mother to the fetus and (2) horizontal transmission in which a two-host life cycle is needed to infect the cattle by ingestion of oocysts shed by the definitive host (Anderson *et al.*, 2000; Bergeron *et al*., 2000). In a study of dogs in dairy farms with known neosporosis prevalence, 23.6% of dogs showed seropositive while only 5.5% of urban dogs were seropositive and there was a relationship of *N. caninum* infection between dog and cattle (Wouda *et al.*, 1999b). *N. caninum* also infects wild foxes in Belgium (Buxton *et al.*, 1997). This indicates that the presence of dogs (or wild canids) on the cattle farms is very important in the horizontal transmission of the disease.

The other important horizontal transmission to be noted is contaminated food and water which could increase the abortion risk associated with neosporosis (McAllister *et al.*, 1998a).

Point source exposure of cattle to *N. caninum* in specific age-group of cows occurring abortion outbreak was reported (McAllister *et al*., 2000; Dijkstra *et al.*, 2002).

Experiments demonstrated that calves could be infected by oral inoculation of *N. caninum* oocysts collected from the infected dog (De Marez *et al.*, 1999) and by feeding colostrum inoculated with tachyzoites (Uggla *et al.*, 1998). Although not proved yet, cow-to-calf transmission through infected or contaminated milk may also be an important route of horizontal transmission. Horizontal cow to cow transmission has not been reported.

The vertical transmission is a major route involved in the spread of *N. caninum* in the cattle herds and there is only a low level of horizontal transmission (Anderson *et al.*, 1997; Davison *et al.*1999a; Davison *et al*. 1999b). The vertical transmission may be as high as 95.2% in a total of 124 seropositive dams and calves (Davison *et al*. 1999b). A similar result was reported in Quebec with 44.4% of seropositivity by vertical transmission (Bergeron *et al.*, 2000). Hietala and Thurmond (1999) suggested that vertical transmission of *N. caninum* occurred mostly in late gestation period rather than postnatal infection and dams remained seropositive throughout their life.

1.2.3 *Pathogenesis*

After the sporulated oocysts are ingested by the susceptible animals, the released tachyzoites invade the variety of host tissues, rapidly grow and multiply destroying the cells. The parasites invade both maternal and fetal tissues through circulation. In pregnant cows the tachyzoite-haboured placenta may lead to abortions depending on the number of parasites, immunostatus of the cow and fetal age (Buxton *et al*., 2002). Multifocal nonsuppurative encephalitis and myocarditis are common findings in histopathological examinations (Barr *et al*., 1991; Wouda *et al*., 1997b). The infection to the neural tissues causes nervous signs in young puppies and calves and some adult dogs. Gross lesions are seldom observed (Dubey and Lindsay, 1996).

1.2.4 *Clinical Symptoms*

Abortion: The *N. caninum* infected cows show no prominent clinical signs. The most visible dramatic effect of neosporosis is abortion and the aborted cows show no clinical illness (Dubey, 1999a). Abortion may occur at any stage of gestation but usually occurs during mid gestation (4^{th} to 6^{th} month of gestation). Aborted fetuses are usually autolysed with no gross lesions and placentas are not retained. Fetuses may die *in utero*, be resorbed, mummified, stillborn, born alive but diseased, or born clinically normal but chronically infected (Dubey, 1999a). Lesions in aborted fetuses are usually only visible upon histological examination and

are located mainly in the brain although pericarditis, myocarditis, hepatitis, pneumonia and nephritis may also be seen (Dubey and Lindsay, 1996; Wouda *et al*., 1997b; Anderson *et al.*, 2000). Abortion may occur in the epidemic (abortion storm) rather than in the endemic (sporadic) form in herds at the rate of 5 to 33% (Wouda *et al*., 1997b; Reichel, 2000).

Abortion storm is defined as a cluster of abortions within a 4-week period involving >15 % of the animals (pregnant cows and heifers) at risk (Moen *et al.*, 1998). *N. caninum* seropositive non-aborting cows had a 2- to 3-fold increased risk of abortion compared with *N. caninum* seronegative cows (Paré *et al*., 1997; Moen *et al.*, 1998). In the study of seropositive descendents, Wouda *et al.* (1998) showed that seropositive cows had a 3-fold increased abortion risk (26.5%) compared with seronegative F_1 cows (8.4%). Repeat abortions are also observed in *Neospora*-infected cows and it is assumed to be due to the recrudescence of the parasite rather than the result of a recent reinfection (Barr *et al.*, 1993; Wouda *et al.*, 1999b). It is not known what triggers the parasite reactivation.

Although early embryonic death may occur due to *N. caninum*, no reports are available at present. It was experimentally proved that zona pellucida of pre-implantation stage embryo could protect invasion of *N. caninum* (Bielanski *et al*., 2002). It is assumed that the *Neospora* transmission through semen is improbable. But in embryo transfer, infection to the fetuses conceived by the seropositive mothers was high while no infection was found in fetuses of seronegative mothers (Baillargeon *et al*., 2001). Therefore, in farms where embryo transfer is practiced should use seronegative recipients.

Calves: Clinical signs are observed in calves only. Congenitally infected calves born alive may be underweight, unable to rise with limbs flexed or hyper-extended or signs of ataxia (Dubey, 1999a). When calves, 6 month after birth, were experimentally infected by feeding colostrum inoculated with tachyzoites, they showed transient fever and blood-stained diarrhea 1-2 weeks after inoculation (Uggla *et al.*, 1998).

Dogs: In dogs these neuromuscular signs can be observed not only in pups (Dubey and Lindsey, 1996) but also in adult dogs (Lorenzo *et al*., 2002). In the later case the dog showed progressive pelvic weakness and difficulty in jumping.

1.2.5 *Effect on Milk Production*

Although no clinical signs, except abortion, are observable, it is possible that several organs such as brain, liver, heart and kidneys, will be affected for their normal physiological functions in infected cows. Consequently, this will lead to a reduced production of cows. Thurmond and Hietala (1997) first reported the reduced production of milk in first-lactation dairy cows infected with *Neospora*. They found that milk and fat production of seropositive

cows was less than seronegative cows (1.14 kg/cow/d and 0.064 kg/cow/d, respectively). The study of Hernandez *et al.* (2001) for more than 4 successive lactations showed that seropositive cows produced 1.27 kg/cow/d less than seronegative cows. After adjustment for the effect of lactation, season, mastitis and lameness, the decrease in milk production was 1.14 kg/cow/d. This led to a loss of $128/cow based on the 305 day mature equivalent production.

1.2.6 *Effect on Weight Gain in Beef Calves*

A very few papers have been reported on the effect of *Neospora* in beef cattle performance. In seropositive beef steers, a significant reduction in average daily gain (0.05 to 0.17 kg/d), impaired feed efficiency and reduction of carcass weight were reported with an estimated loss of $15.62/calf during post weaning period (Barling *et al.*, 2000; Barling *et al.*, 2001).

1.2.7 *Diagnosis*

The prior importance for the diagnosis of neosporosis may be by tracing the records and history. There is highly significant association between seropositivity and history of abortion (Atkinson *et al*., 2000). As there are no distinct clinical signs of neosporosis in cattle except abortion, it may not help very much in the diagnosis. Mostly the help of diagnostic laboratories may facilitate in identifying neosporosis.

Immunohistopathology: For a definitive and confirmative diagnosis of neosporosis, histological and immunohistochemical test are required. The samples of aborted fetuses (brain, heart and liver) and placentas are required for the test. But in most cases the aborted fetuses are autolysed and not suitable for the normal routine diagnostic procedures. It is very common to see only a few *N. caninum* organisms in tissues with HE stained sections. Therefore, the use of immunohistochemical techniques is required for a definitive diagnosis (Dubey, 1999b). The fetal brain tissue is most commonly used for the diagnosis and it usually consists of necrosis and nonsuppurative focal inflammation (Dubey, 1999a). In a study of tissues from aborted fetuses with confirmed *Neospora*-infection, tachyzoites are identified immunohistochemically in 85% of the brains, 14% of the hearts, and 26% of the livers; tissue cysts were found only in the brains (Wouda *et al*, 1997b).

Serology: This is the most commonly used method of diagnosing neosporosis. One of various serological tests is direct agglutination test (DAT) (Romand *et al*., 1998; Packham *et al*., 1998). The advantage of DAT is that it can test the serum of any species without secondary antibodies and special equipment. In addition, it is easy to use and relatively cheap. Therefore,

this test may become the choice for the diagnostic purpose. Although they are cheaper and easier, extensive use of the technique has not been found yet.

Indirect Fluorescent Antibody Test (IFAT) has been used as a gold standard with high specificity and sensitivity. It has no cross-reaction with the most related parasite *T. gondii* (Barr *et al*., 1995) but it is a time consuming test for large samples. At present, enzyme linked immunosorbent assay (ELISA) technique is widely used. It is very useful for testing large samples. Some modified tests have also been developed to improve the sensitivity and specificity of the tests; e.g., the use of immune stimulating complex ELISA (iscom ELISA) (Björkman and Lunden, 1998; Slotved *et al*., 1999), monoclonal antibody based competitive inhibition ELISA (CI-ELISA) (Baszler *et al.*, 1996; Baszler *et al.*, 2001) and IgG avidity ELISA (Björkman *et al.*, 1999). The use of iscom ELISA is also recommended for screening specific antibodies against *N. caninum* in the fetal fluid (Slotved *et al*., 1999). Antibodies against *N. caninum* could be detected not only in the sera and milk (Björkman and Lunden, 1998; Ooi *et al*., 2000; Chanlun, 2002) but also in the vaginal secretions and in the saliva of cattle (Ooi *et al.*, 2000).

Until now, IgG avidity ELISA is the only test which can distinguish between recent and chronic neosporosis (Björkman *et al*., 1999). In experimentally *Neospora*-infected cattle, the IgG avidity was 9-18% three weeks after infection and it had increased to 58-76% twenty-four weeks later. In naturally infected cattle for more than 6 months, all had an avidity value of greater than 50%. The test is of value in epidemiological studies in *Neospora* infection in cattle. One common problem of diagnosis using serological tests is to assess a definitive cut-off titer. This is because the titer and absorbance values depend on the antigen composition, secondary antibodies, age of animals and other reagents (Dubey, 1999a).

Polymerase chain reaction (PCR) is the most sensitive method and becomes more common in diagnosing *N. caninum* infections. The brain is the major organ targeted for detecting parasite DNA. It is interesting that the attempts to detect the parasite DNA in the blood of infected cattle, even with PCR, the most sensitive test, were unsuccessful. This may be because the parasites present in the blood are very small in numbers and they remains for a very short period (Maley *et al*., 2003). Recently, the parasite DNA was detected from the whole blood, leukocytes and lymphocytes, but not from the serum, of the infected 4 to 5 month pregnant heifers in New Zealand (Okeoma et al., 2004).

1.2.8 *Control and Prevention*

Although there were successful treatments for neosporosis in dogs with clindamycin, sulphonamides and/or pyrimethamine (Barber and Trees, 1996), no report has been found yet for the treatment in the livestock animals. Experimentally, intracellular multiplication of *N. caninum* tachyzoites can be effectively inhibited by artemisinin, an anticoccidiosis drug, in cell cultures without toxicity to the host cells (Kim *et al*., 2002).

With respect to the preventive purpose vaccines are still at their early stage of research and some vaccines are under field trials (Barling *et al*., 2003; Romero *et al.*, 2004). Lack of effective drugs for the treatment and commercially available effective vaccines for prevention of *Neospora* infection becomes the most important problem in the control and preventive work of the disease until now. Although a killed *Neospora* vaccine has been introduced by Intervet Company, its efficacy is still to be verified. The danger and spread of the disease may be even more problematic in countries where the disease occurrence has not been identified yet. However, a considerable measures of preventive and control methods can be achieved mainly by:

1. Selective culling of infected or seropositive animals in the herd (but this may not be practical in farms with high seroprevalance) and preventing the risk of introducing infected replacement cattle,
2. careful removal of aborted fetuses and associated materials as placentas and fetal membranes, and
3. serotesting farm dogs and removal of positive ones from the farm and taking care of the possible fecal contamination of feed and water by infected dogs.

1.3 Neosporosis and Thailand

As discussed, neosporosis is an important disease affecting cattle production by abortions, repeat abortions, still-birth and birth of weak calves. Thailand has a large cattle population (dairy and beef) of about 6.3 millions and 1.6 million buffaloes (DLD, 2003). These animals involve in the important economic sector of the country through milk and meat production. But little is known about neosporosis in these animals. In cattle, the seroprevalence of *Neospora caninum* was first reported by Suteeraparp *et al.* (1999) and in farm dogs by Kyaw *et al.* (2004). The seroprevalence ranges from zero to 70% (mean = 6.9%), depending on the area and season (Suteeraparp *et al*., 1999; Kashiwazaki *et al*., 2001; Chanlun *et al*., 2002). These studies indicate that the neosporosis occurs in dairy cattle throughout the country. There are no reports of abortion outbreaks related to *Neospora* infection. *Neospora* seroprevalence in farm dogs is very low (only 1.22%; 1/82) and the presence of dog on the farm is not related to

the herd infection (Kyaw *et al*., 2004). As the areas reported in Thai studies have no wild canids, which are assumed as definitive hosts, there may be other definitive hosts responsible for the *Neospora* transmission.

1. 4 Rationale and objectives

Concerning with *Neospora* only a few studies have been done in Thailand; they all are mainly about the seroprevalence studies. Only one report described the identification of *Neospora* parasite by IHC in the placenta of a *Neospora* seropositive cow (Kyaw *et al*., 2003). But it was not tested and confirmed by other means like PCR or not tested for other related parasites as *Toxoplasma gondii*. It is not known about reproductive performance of cows in the *Neospora*-exposed dairy herds, especially of abortion status related to this parasite. The ability to estimate the abortion risk from the serological status will be of importance to put in place any preventive and control measures.

The aims of this study were: (1) to identify *Neospora caninum* in the aborted fetal tissues, and to confirm by histopathological, immunohistochemical (IHC) and PCR methods with an attempt to establish a guideline for the isolation of *Neospora* parasite from the brains of aborted fetuses or calves of seropositive cows and (2) to investigate the impact of *Neospora* parasite on the reproductive performance (the number of artificial insemination (AI) per confirmed conception, age at first service, first service conception rate, age at first calving, calving to first insemination, calving to conception, calving intervals, retention of placenta, stillbirth and abortions) of seropositive cows and (3) to estimate abortion risk of seropositive cows in comparison to the seronegative cows.

CHAPTER II

IDENTIFICATION AND ESTABLISHMENT OF A GUIDELINE FOR THE ISOLATION OF *Neospora caninum* FROM ABORTED/CALF TISSUES OR COWS

2.1 Introduction

This chapter involves two studies. The first study was to identify *N. caninum* in the aborted fetal tissues collected from the dairy farms and relate to the seropositivity of dams and abortions. The second one was to establish a guideline for the isolation of *N. caninum* from the brains of aborted fetuses or calves of seropositive pregnant cows.

2.2 Identification of *Neospora caninum* in the aborted fetal tissues

2.2.1 Materials and methods

2.2.1.1 *Sample Collection*

The tissue samples (brain, heart, liver and kidney) of the aborted fetuses and placentas were collected from two seropositive dairy farms (Bangbung and Nongulam) in Chonburi, having about 380 and 230 cows each, in Chonburi during 2002 to 2004. The tissues were preserved in 10% neutral buffered formalin for histopathological (HE staining) and immunohistochemical (IHC) examinations. Maternal blood and fetal blood (or body fluids) were also collected to detect *Neospora* antibody. A portion of each fetal brain collected was kept at -70°C for identification of the *N. caninum* by polymerase chain reaction (PCR) method. The brains from seropositive fetuses or the fetal brains of seropositive cows were attempted to culture in vero cells. Blood samples of the cows and heifers were collected to check *Neospora*-prevalence in July 2003.

2.2.1.2 *Serological Test*

Indirect fluorescent antibody test (IFAT) was used to detect *Neospora* antibody. Antigen coated slides were prepared from *Neospora caninum* (NC1, provided by J.P. Dubey, USDA-ARS, USA) cultured and maintained in the vero cells. Fluorescein labelled goat anti-bovine IgG (KPL, Maryland, USA) was used as secondary antibody. The sera were screened at 1:200 dilution for cows (Dubey and Lindsay, 1996) and 1:25 dilution for fetuses (Wouda *et al*., 1997a). All positive sera were further tested for their titers. The serum from an aborting cow which reacted with cELISA (91.9 % inhibition value) and IFA (1:3200) was used as positive control. The serum of non-aborting cow which does not react with cELISA and IFAT was used

as negative control. The positive and negative control wells were added in every test. The positive sera were the sera which show clear whole tachyzoite fluorescence (Fig 2).

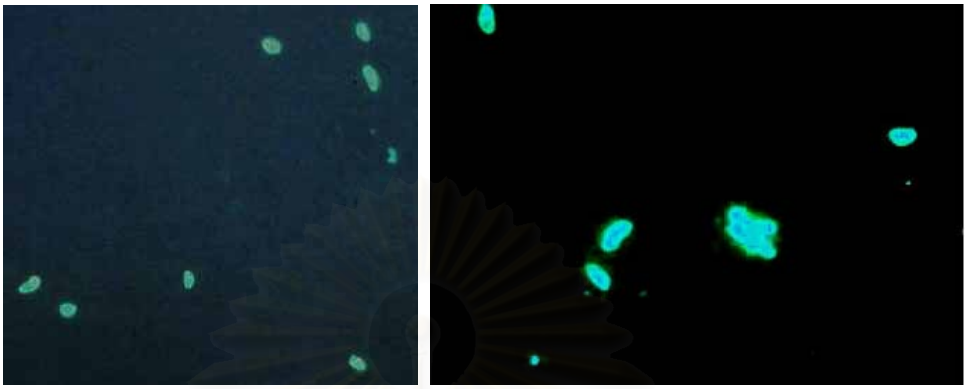

Fig. 2. *Neospora caninum* tachyzoites positively reacted by IFA showing whole peripheral fluorescence.

The antibody to the related parasite, *T. gondii*, was also checked by using direct agglutination test (Toxo-screen, Bio Merieux, Lyon, France) at a dilution of 1:100 (Björkman *et al*., 1996). Twenty-five microliters of the diluted sample and control sera were placed into the wells of microtitration plates according to the prepared records. To each well, a 25 µl of 0.2 mol/l 2-mercaptomethanol was added. Then, 50 µl of diluted toxoplasma antigen suspension was placed into every well. A well for antigen control was also included for checking negative control. In the antigen control well, 25 µl 2-mercaptoethanol, 25 µl PBS and 50 µl of antigen suspension were included. The contents were homogenized by shaking the plates with a shaker for a few minutes. The plates were left still at room temperature for 18 hours. The reading was made as follows:

Negative reaction and antigen control: sedimentation of the toxoplasma in a button or ring.

Positive reaction: agglutination of the toxoplasma in a mat covering about half of the well base.

2.2.1.3 *Direct Culture in Vero Cells*

For isolation of *Neospora* parasite, trypsinized brain homogenates of the fetus was cultured in 24 hour vero cell monolayers as described by Yamane *et al*. (1997) (Appendix A). Briefly, 12 g of brain tissue was homogenized in 50 ml of PBS containing 0.25% trypsin and 0.025% EDTA, filtered with sterile gauze, incubated at 37^0C for 45 min, centrifuged at 300 g for 10 min, washed with PBS 3 times and resuspended in 20 ml of RPMI 1640. One ml each of

the suspension and 5 ml of RPMI 1640 with antibiotics (100 IU Penicillin and 100 µg streptomycin/ml) were added into the vero cell monolayer (25 cm^2 flasks) and incubated at 37^0C for 4 hours. The monolayer was washed with PBS three times and fresh RPMI was added and incubated at 37^0C, 5% CO_2 incubator. The flasks were checked daily and the medium was changed twice weekly.

2.2.1.4 *Histopathology and Immunohistochemistry*

Formalin-fixed, paraffin-embedded, tissue sections (4-5µm thickness) were stained with a routine hematoxylin and eosin stain (HE) (Appendix B). An avidin-biotin complex (ABC) method was used for immunohistochemical staining to detect NC parasites (Appendix C). Briefly, formalin-fixed, paraffin-embedded tissues, including a positive control (infected goat heart provided by McAllister, Illinois, USA), were cut and deparaffinized. The endogenous peroxidase activity was blocked with 0.3%H_2O_2 in methanol. For antigen retrieval, the slides were placed in a jar of Tris EDTA (pH9) and heated in a microwave oven for 10 min. Non-specific antigens were blocked with 1% bovine serum albumin. Primary rabbit anti-NC serum (provided by McAllister) was applied to the slides at a dilution of 1:10,000 in phosphate buffer saline (PBS) and kept overnight at 4^oC. The biotinylated goat anti-rabbit IgG antibody (Dako, Denmark) at 1:400 dilution with PBS, was used as a secondary antibody. After the application procedures with the ABC kit (Dako) and 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, USA) the slides were counterstained with Mayer's hematoxylin. PBS, instead of primary antibody, was used for the negative control slides. The histopathological and immunohistochemical staining were examined under a light microscope.

2.2.1.5 *PCR*

Gene extraction from the brain tissues was done using QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacture's instruction (Appendix D). Published *N. caninum*-specific primers, Np21 (5'-GTGCGTCCAATCCTGTAAC-3') and Np6 (5'-CAGTCAACCTACGTCTTCT-3') (Yamage *et al*., 1996) were used for amplification. The DNA extracts were amplified in 25µl reaction volumes containing 10mM Tris-HCL pH 8.4, 50 mM KCl, 10 pM of each primer, 0.2 mM of each dNTP and 1 U Platinum *Taq* DNA polymerase (Invitrogen, Life Technologies, Brazil). DNA extract from cultured NC1-parasites was used as positive control and DNA extract from the uninfected vero cell culture as negative control. A 35 cycle amplification consisting of an initial denaturation (94°C, 2 sec), denaturation (95°C, 30 sec), annealing (56°C, 30sec) and extension (74°C, 45 sec) with a final extension (75°C, 5 min) was done using Sprint Thermal Cycler (Thermobaid, Thermo Electron

Corporation, Waltham, MA). The PCR products (10 μl each) were analysed by electrophoresis on 2% agarose gel stained with ethidium bromide and viewed under UV light.

2.2.2 Results

2.2.2.1 *Samples*

A total of 22 aborted fetuses (15 from Bangbung and 7 from Nongulam; Appendix E) were collected between July 2002 and March 2004. The organs or parts of some fetuses were not available at time of collection, especially visceral organs. Some of the collected fetuses with missing parts and a part of placenta were shown in Fig 3. The gestation age of the aborted fetuses ranged from 3 to 8 months.

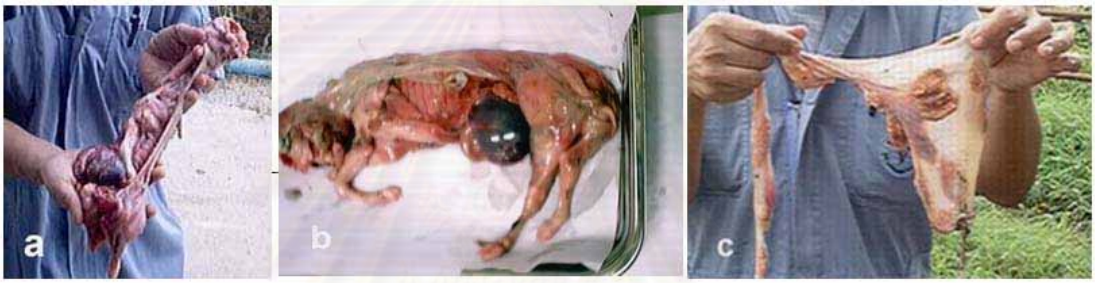

Fig. 3. (**a** and **b**) Aborted fetuses with missing parts and/or visceral organs. (**c**) A part of the placenta from an aborted cow.

2.2.2.2 *Serology*

Only one of 22 aborting cows was seropositive to *Neospora* with a titer of 1:400. This cow was from Bangbung. The serum or body fluid of its fetus was not available and all visceral organs were lost. The sera of the other fetuses and their dams did not react with *Neospora* (<1:25 and <1:200, respectively). All sera of cows and their fetuses also did not react with the direct agglutination test for *T. gondii*. The *Neospora* seroprevalence of the two farms is shown in Table 1. The serological titers ranged from 1:200 to 1:1600.

Table 1. *Neospora* serostatus of two farms in Chonburi*.

Farm	# cows tested	# seropositive					Seroprevalence
		1:1600	1:800	1:400	1:200	total	
Nongulam	381	2	7	4	11	24	6.3%
Bangbung	232	2	2	4	6	14	6.03%
Total	613	4	9	8	17	38	

* Blood collected on: July 2003.

2.2.2.3 *Culture, Histopathology and Immunohistochemistry*

Only one fetal brain (No.251) of seropositive cow was cultured in vero cells. Parasite growth was not observed in the vero cell culture until 60 days. As all visceral organs of this fetus were lost, only the brain was histopathologically checked. *Neospora* tachyzoites were not found in the HE stained sections but diffuse non-suppurative mononuclear cell infiltration was observed in the parenchyma (Fig. 4a). *N. caninum* tachyzoites were immunohistologically identified and they were found in the paranchymal and endothelial cells of the blood vessels (Fig. 4b and 4c). Tissue cyst was not observed in any sections.

2.2.2.4 *PCR*

Although the *Neospora* organisms were labelled with IHC in the brain of one fetus, DNA was not amplified from it and all of the other fetal brains by PCR.

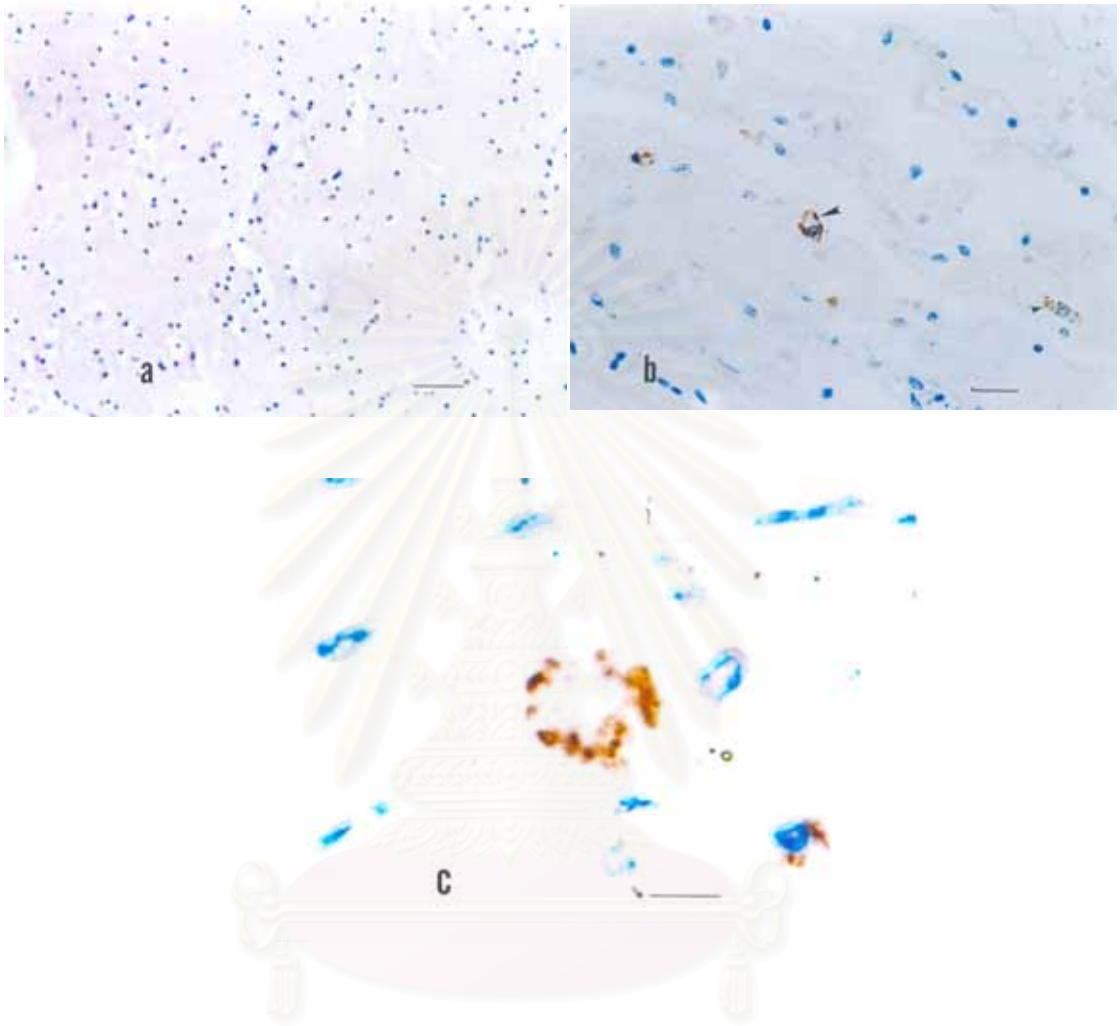

Fig. 4. HE and IHC staining of the brain of fetus No. 251. (**a**) The brain showing diffuse non-suppurative necrosis, HE stain (Bar = 50 μm). (**b**) The brain of the same fetus showing IHC labelled tachyzoites in the endothelial lining of small blood vessels (large arrow head) and in the parenchymal cell (small arrow head). (Bar = 20 μm). (**c**) Tachyzoites in the endothelium of a small blood vessel at higher magnification (Bar = 20 μm).

Discussion

Absence of body parts and visceral organs in some fetuses may be due to the farm dogs which ate them at the time of abortion. Generally the tachyzoites and/or tissue cysts were most frequently observed in the IHC labelled brain tissue sections of infected fetuses (Dubey and Lindsay, 1996). Rodrigues *et al*. (2004) could not amplify the NC DNA from the six seropositive buffaloes. Even the different portions from the same brain tissue were not always PCR positive, indicating that parasites are not uniformly distributed in tissues (De Marez *et al*., 1999). In this study, the identification of *Neospora* organisms by IHC in the brain of one aborted fetus and seropositivity of its dam indicated that the fetus was congenitally infected. The reason of PCR negativity of this fetus may be due to lack of organisms in the portion of the brain tested. The finding of diffuse non-suppurative mononuclear infiltration was consistent with our previous report where similar lesions were observed in the placenta of an aborting cow (Kyaw *et al*., 2003). But it was in contrast with the other reports where multifocal non-suppurative necrotizing areas were observed (Barr *et al*., 1995; Wouda *et al*., 1997b). Therefore, it is possible that both diffuse and focal non-suppurative necrosis may be observed in the *Neospora* infected tissues. The gestation age, 5 months, of the *Neospora*-positive fetus was in agreement with the other reports (Dubey and Lindsey, 1996). As the two farms from which the fetuses were collected have been annually vaccinated against bovine diarrhoea virus disease (BVD), infectious bovine rhinotrachitis (IBR), bovine parainfluenza (PI3) and bovine respiratory syncytial virus disease (BRSV), it was more likely that the IHC positive fetus was aborted due to the *Neospora*-infection. This is the first report of congenitally *Neospora*-infected abortion in Thailand. Moreover, as all of the other aborting cows (95.5%; 21/22) and their fetuses were seronegative to *Neospora caninum*, it should be noted that most of the abortions were due to other unidentified factors rather than *N. caninum*.

In conclusion, neosporosis is not a significant cause of abortions in these farms as evidenced by low seroprevalence and low percentage of *Neospora*-infected abortions.

2.3 The establishment of a guideline for the isolation of *Neospora* parasite from the calves or aborted fetal brains of seropositive cows

2.3.1 Materials and methods

2.3.1.1 *Cows*

Cows from a dairy herd (Chonburi; section 2.2.1.1) were tested for the antibody to *Neospora* 1 to 2 months before AI using indirect fluorescent antibody (IFA) test. Four seropositive cows (Nos. 203, 2156, 1118 and 742), after pregnancy was confirmed, were moved to and confined in a shed at Nakhon Pathom Animal Hospital. They were monitored to observe whether normally calved or had abortions. The shed was fenced with wire-mesh to protect access of dogs. They had reciprocal titers of 1600, 800, 800 and 400 respectively. Two cows (Nos. 203 and 472) had abortion history and cow No. 472 was previously ELISA positive (Kyaw, 2003). After calving, cows were euthanized within 3 to 4 weeks and their calves within 2 weeks. Their brains and various tissue samples (heart, lung, liver, kidney, muscle and placentas) were collected and preserved in 10% neutral buffered formalin to detect the presence of *Neospora* parasites by histopathological and immunohistochemical (IHC) examinations. A portion of the brain from paraventricular area (Kim *et al.*, 2000) of each cow and calf was used for culture in vero cells and bioassay in hamsters. Some portions of brain were also kept at -20^{0}C for DNA extraction. The sera of dams and the calves (precolostral) were collected at the time of parturition to check the NC antibody status.

2.3.1.2 *Serological Test*

Cow sera and precolostral blood of calves were tested for NC antibody using IFA. The antibody to the most related parasite, *T. gondii* was also checked by using direct agglutination test (as described in section 2.2.1.2).

N. caninum antibodies were also detected using competitive enzyme-linked immunosorbent assay (c-ELISA) technique with commercially available test kits (VMRD, Inc., USA). Sera, reagents and 96 well *N. caninum* antigen-coated microplates were brought to room temperature before testing. Eighty microliter of each sample sera, positive control (2 wells), and negative control (2 wells) sera were placed in the wells of transfer plates according to the prepared setup records. Using a multichannel micropipette set at 50 μl, the samples were transferred to the *N. caninum* antigen-coated microplates and incubated for 1 hour at room temperature (21-25°C). The plates were gently mixed by shaking while incubating. Then the wells were emptied. The remaining sera and controls were removed by striking the inverted

wells 4 times on a clean paper towel. Each well was washed 3 times with wash solution. After every wash the plates were struck 4 times on the paper towel to remove residual wash solution. Fifty microliter of diluted, horseradish peroxidase-labelled *N. caninum*-specific monoclonal antibody was added to each well and the plates were incubated for 20 minutes at room temperature. The plates were washed 3 times as mentioned above. To each well 50 µl of substrate solution was added, mixed and incubated for 20 minutes at room temperature covering the plates with aluminium foils. After incubation, 50 µl of stop solution was added to each well and gently mixed by tapping the side of the plates several times. Immediately after adding the stop solution the plates were read on a microplate reader (Titertek Multiskan Plus, Finland) set at a wave length of 650 nm optical density (OD). The percent inhibition of antibodies to the antigens was calculated by using the formula:

% Inhibition = 100 – [(sample OD x 100)/(mean negative control OD)]

The samples with the values of $\geq$ 30% inhibition were regarded as positive and those with the values of < 30% inhibition were regarded as negative.

2.3.1.3 *Direct Culture in Vero Cell Monolayers*

Isolation of *Neospora* parasite from the trypsinized brain homogenates of dams, calves and hamsters was done as described in the section 2.2.1.3.

2.3.1.4 *Bioassay* in *Hamsters*

The brain homogenates of two cows (No. 203 and 2156) and of their calves were bioassayed using 8 to 12 weeks old Golden hamsters (*Mesocricetus auratus*) bought from the laboratory animal center of Mahidol University. For each brain of cows and calves, 6 hamsters (2 control and 4 inoculated with brain homogenates) were used. In the treatment group, one ml of homogenised brain tissue suspended in the antibiotic PBS (100 IU penicillin and 100 µg streptomycin/ml PBS) were injected intraperitoneally into the hamsters. For the control animals, antibiotic PBS without brain homogenate was inoculated. The hamsters were treated with dexamethasone sodium phosphate (LBS laboratory Ltd, Bangkok, Thailand) at a concentration of 10µg/ml in drinking water ad lib 10 days before brain homogenate inoculation (Romand *et al*, 1998). They were euthanized at 5 weeks post inoculation (Uchida *et al.*, 2003). In each treatment group, one half of the brain from each hamster, after aseptically collected, was pooled and processed as mentioned above. The brain homogenate was cultured in the vero cell monolayers. Cell cultures were observed daily for the presence of parasites for 60 days (Stenlund *et al.*, 1997). One-fourth of the brain was kept at -20^{o}C for DNA extraction. The rest

of the brain and other organs (heart, lung, liver and kidney) were preserved in 10 % neutral buffered formalin for histopathological and immunohistochemical examinations.

2.3.1.5 *Histopathology and Immunohistochemistry*

HE and immunohistochemical staining of tissue sections of cows, calves and hamsters were done as described in section 2.2.1.4.

2.3.1.6 *PCR*

Gene extraction, DNA amplification and electrophoresis of the brain tissues (cows, calves and hamsters) were done as described in section 2.2.1.5. PCR products were purified and sequenced using ABI 3100 genetic analyzer, USA. Nucleotide sequences were aligned by using Clustal-W and BioEdit Sequence Alignment Editor 7.0.1.

2.3.2 Results

The summary of the experimental results of the cultural, histopathological and PCR are shown in table 2.

2.3.2.1 *Cows*

All cows were normal throughout the experimental period and they gave birth to healthy calves. A cow (No. 472) died early in the morning two days after parturition without signs of sickness. The tissues samples were taken within 12 hours after death. No gross lesions were observed during the sample collection; the cause of death was unknown.

2.3.2.2 *Serology of Cows and Calves*

All sera of cows and precolostral bloods of calves were not reacted with the direct agglutination test for *T. gondii*. Although cows were seropositive for *N. caninum* before breeding, they all were seronegative by IFA at calving (< 1:200). Further testing at lower dilutions showed that 2 cows (Nos. 203 and 2156) had a titer of 1:100 but by ELISA cow 2156 had an inhibition value of 69.3%. Calf 2156 had a precolostral antibody titer of 1:100 and an ELISA value of 73.1% inhibition but the other calves were seronegative (< 1:25).

Table 2. Cultural, histopathological, serological and PCR results of cows and their calves.

Dam and calves		Brain				Serology :IFA		Bioassay with hamsters
		Culture	H&E	IHC	PCR	Before breeding	At calving	
203	Dam	-	-	-	-	1:1600	1:100	-
	Calf	-	-	-	-		<1:25	-
2156	Dam	-	-	-	-	1:800	1:100	-
	Calf	-	-	+ *	+		1:100	**
1118	Dam	-	-	-	-	1:800	<1:25	Not done
	Calf	-	-	-	-		<1:25	Not done
472	Dam	-	-	-	-	1:400	<1:25	Not done
	Calf	-	-	-	-		<1:25	Not done

* A tissue cyst was identified in the brain.

** The brains of 2 hamsters (H2 and H3) inoculated with the brain homogenates of the calf were PCR positive. And multifocal non-suppurative encephalitis was observed in the brain of H3.

2.3.2.3 *Culture and Bioassay*

All hamsters showed no signs of clinical illness throughout the experiment. Parasite growth was not found in any of the vero cell cultures inoculated with the brain homogenates of cows, calves and hamsters until 60 days of incubation.

2.3.2.4 *Histopathological and Immunohistochemical Test*

With one exception, all tissue sections of cows and calves (brain, heart, lung, kidney, muscle and placenta) examined with H&E and IHC showed no characteristic lesions of *Neospora* organisms. A tissue cyst, measuring 27 μm in diameter and having a characteristic thick cyst wall was labelled by IHC in the brain of the calf-2156 (Fig. 5a). There was no

inflammatory reaction around the cyst. The brain of one hamster (H3) inoculated with the brain homogenate of the *Neospora*-positive calf showed multifocal non-suppurative inflammation by the accumulation of mononuclear cells (Fig. 5b). Microscopic sarcocysts were observed in the skeletal muscle of all cows. These cysts were not reacted with *Neospora* antibody by IHC.

2.3.2.5 *PCR*

The amplified DNA extracts of the brains of a calf (No.2156) and 2 of four hamsters (H2 and H3) inoculated with the brain homogenates of that calf were shown in Fig. 6. NC DNA could not be amplified from the brains of all cows, the other 3 calves and all hamsters inoculated with the brain homogenates of cow No. 203 and its calf and 2 hamsters (H1 and H2) of cow No.2156 (see Table. 1).

Nucleotide sequence of the PCR-positive calf is shown with the reference strain NC1 (accession number AY665719) (Fig. 7). From the targeted 328 bp, 225 were identical, except at 2 positions as shown in the figure. The DNA sequence was designated as Thai-B1 and has been deposited in the GenBank database under the accession number AY941177.

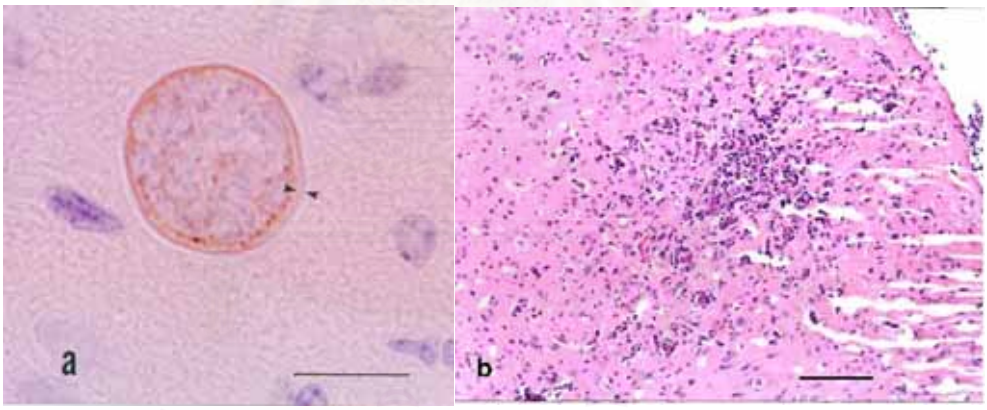

Fig. 5. (**a**) A tissue cyst identified by IHC in the brain of calf No. 2516. Cyst wall is thick and no inflammatory cells are present around the cyst. (bar = 15 μm). (**b**) Focal non-suppurative encephalitis in the brain of hamster H3. (bar = 100 μm)

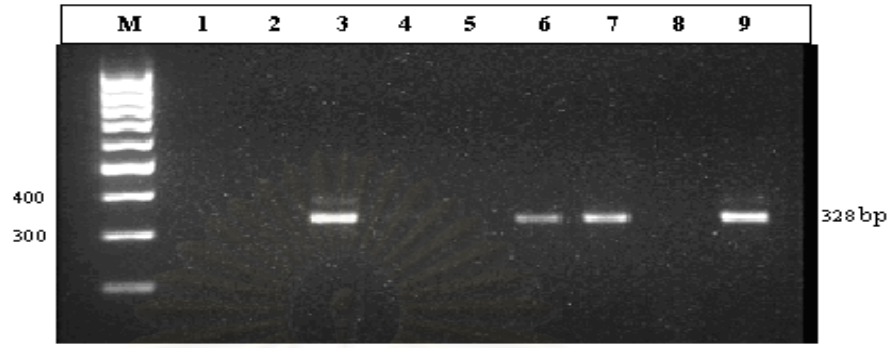

Fig. 6. DNA amplified from the brains of cow No. 2156, its calf and hamsters inoculated with brain homogenates of that calf. M, 100 bp DNA marker; lane 1, negative control; lane 2, dam; lane 3, calf; lane 4 , control hamster not inoculated with brain homogenate; lanes 5 to 8, hamsters inoculated with the brain homogenate of the calf; lane 9, positive control.

```
Thai-B1  ATGCGGACGTGTCGTTGTTGGGCGCAGCCTGCGGCAGCAAGGCTCCTTTTTTGTTTGTGG
NC1      ............................................................

Thai-B1  CTATAGTGTGTGAACGGGTGAACCGAGGGAGTTGGTAGCGGTGAGAGGTGGGATACGTGG
NC1      ............................................................

Thai-B1  TTTGTGGTTAGTCATTCGTCACGTTGAAATGAGCCTGCGTCAGGGTGTGGACAATGTGTC
NC1      ............................C.....................G......

Thai-B1  AATGATACTTATCCAGAGTTCAGTGTTCTGTGTTGAGGCAACACC
NC1      ....................................
```

Fig. 7. Nucleotide sequence alignment of *N. caninum* by PCR product of calf 2156 with the reference strain NC1 (accession number AY665719). Dots indicate nucleotides identical in both sequences. The nucleotide sequence was designated as Thai-B1 (Genbank accession number AY941177).

2.3.3 Discussion

In Thailand there are a number of reports on the serological evidences of *Neospora* infection in dairy cattle (Suteeraparp *et al*., 1999; Kashiwazaki *et al*., 2001; Chanlun *et al*., 2002; Kyaw *et al*., 2004) and a report on the serologically *Neospora* related abortions in cattle (Suteeraparp *et al*., 1999). Only one study mentioned about histopathological lesions in aborted fetuses and *Neospora*-organisms by IHC in the placenta of a seropositive aborting cow (Kyaw *et al.*, 2003). The present study is the first confirmed report of *Neospora caninum* parasite in a naturally and congenitally infected calf by serology, PCR and bioassay in Thailand. The presence of a tissue cyst (positive by IHC) in the brain of the calf (No.2156) indicates the congenital infection. NC-specific DNA amplification by PCR from the calf brain and from the brains of hamsters inoculated with the brain homogenate of that calf confirmed transplacental infection. Moreover, the primer pairs (Np21 and Np6) do not amplify *N. hughesi* (Basso *et al*., 2001) or the other related parasites *T. gondii*, (Yamage *et al*., 1996; Gondim *et al*., 2001). Although a tissue cyst was found, lesions or organisms were not detected by HE and IHC in any tissue tested. There were similar findings where lesions, tachyzoites or tissue cysts were not identified in the brains of the healthy congenitally infected calf or of the aborted fetus (e.g., Yamane *et al*., 1997; Davison *et al*., 1999c) and experimentally infected calves (De Marez *et al*., 1999) from which NC organisms were isolated or NC DNA was PCR-amplified. In buffaloes, Rodrigues *et al*. (2004) could not amplify the NC DNA from the seropositive male buffaloes. The rarity of the organisms in the tissues was evident. This might be one possible reason that the isolation of the parasite was not successful in the present study. The suggested predilection site for NC parasite in the brain of calf (Kim *et al*., 2000) and dog (Umemura *et al*., 1992) is the paraventricular region. The result of the present study indicates that the paraventricular area of the brain in cattle may not be the predilection site for NC parasites. As many successful isolation studies did not mention the specific part of the brain used, it seemed that the parasites were distributed in the unspecified locations in the brain.

Because all cows became seronegative at the cut-off value of 1:200 and gave birth to healthy seronegative calves, it was likely that parasite reactivation did not occur during pregnancy in the three cows. This fact, for these three cows, may be explained by the negative PCR and histological results from their calves and hamsters inoculated with the brains of those calves. The reason why the dam of *Neospora*-positive calf was seronegative was not explainable; it may be due to a few numbers of reactivated parasites and undetectable amount of antibody. In the study of Williams *et al*. (2003) five naturally infected pregnant cows, even after challenging with NC parasite, gave birth to normal calves two of which were seronegative

and parasites were not detected in any tissues by PCR. There are reports that seropositive cows may give birth to seronegative calves and seronegative cows give birth to seropositive calves (Paré *et al*., 1996; Schares *et al*., 1998; Davison *et al*., 1999c). Majority of congenitally infected calves were born healthy (Paré *et al*., 1996). Davison *et al*. (1999c) successfully isolated the parasites from an infected stillborn calf whose dam was seronegative (by ELISA) at calving. This indicates that seronegativity does not guarantee the absence of organism in the animal or congenital infection. As the cows were confined and access of dogs to the shed was protected, the infection of the positive calf must be due to the recrudescence rather than new reinfection. As the calf was euthanised 11 days of age, it would not be possible for a thick walled cyst to form this rapidly if the calf had become infected after birth.

Changes in antibody titers of NC-positive cows are contradicting. Hietala and Thurmond (1999) suggested that seropositivity may remain long, even for life. In a long term study in Sweden, seropositive cows became seronegative but the percentage was very low (Stenlund *et al*., 2003). In another study, all seropositive cows remained seropositive after 6 to 12 months recheck (López-Gatius *et al*., 2004). But in another report, antibody titers declined to the low or undetectable levels within 2 months after abortion (Cox *et al*., 1998). In a study in naturally infected cows, antibody levels decreased to an undetectable level within 5 months after abortions (Conrad *et al*., 1993). In normal calving seropositive cows antibody level was at its peak 4 to 5 months before parturition and decreased 2 months before parturition (Stenlund *et al*., 1999). Antibody levels can also be changed between positive and negative (Hietala and Thurmond, 1999; Stenlund *et al*., 2003). In pregnant cows experimentally infected with NC oocysts, antibody levels remained elevated only for 12 to 14 weeks (Trees *et al*., 2002). Cows with infected offspring had continuously rising antibody titers, whereas cows with uninfected offspring had falling titers after an early peak (Gondim *et al*., 2004b). In general antibody titers in aborting cows were higher than in non-aborting cows (Dubey *et al*., 1997; Conrad *et al*., 1993). In the present study cows became seronegative within 10 to 12 months. Because the fact that not all calves born from the seropositive cows were infected, it is obvious that reactivation does not occur in every pregnancy. This is in agreement with the results of Williams *et al*. (2003). It is still unclear when and what triggers the reactivation of the parasites in pregnant cows.

As mentioned, all 4 cows in this study had moderately high antibody titers before breeding, but they each became seronegative by the time of calving. This unexpected finding has great potential importance. In some studies, cows have been classified as being seronegative (uninfected) or seropositive (infected) by using serology at the time they gave birth (e.g., Davison *et al*., 1999a). By only performing serology at calving, investigators may

fail to detect infected cows that have (at least temporarily) become seronegative. In another study, only cows with a tremendously high cut-off titer (1:5,120) were used to demonstrate the occurrence of transplacental transmission, with 100% (20/20) of infected cows giving birth to infected calves (Anderson *et al*., 1997); however, if studies would include cows with low titers, then the rate of transplacental transmission would probably be much lower. The seropositive cows may at times become seronegative (Jenkins *et al*., 1997; Cox *et al*., 1998; Stenlund *et al*., 2003; López-Gatius *et al*., 2004). One report described remarkably high negative seroconversion rate ranging from 28.2 to 42.4% within a year; about 20% of them were strongly seropositive at the beginning (Pan *et al*., 2004). Therefore, classification of cows as uninfected should not be based on serology at only a single point, but should be based on consistently seronegative results at several time points.

Only 1 of 4 calves in this study was congenitally infected as evidenced by serology, PCR and bioassay. This suggests that this pattern may be a common occurrence and actual transplacental infection may be lower than the previous reports. On the other hand, we should not underestimate the potential of horizontal transmission by canids. Even before the discovery of dogs as definitive hosts (McAllister *et al*., 1998a), evidence was accumulating of the importance of dogs related to the prevalence of bovine neosporosis (Paré *et al*., 1998; Bartels *et al*., 1999). More and more reports have been accumulating expressing the presence and numbers of dogs on the farm as a very important risk factor and strong association with herd seroprevalence or with the *Neospora*-related abortions (Sawada *et al*., 1998; Ould-Amrouche *et al*., 1999; Mainar-Jaime *et al*., 1999; Wouda *et al*., 1999b; de Souza *et al*., 2002; Dijkstra *et al*., 2002; Guimarães *et al*., 2004). Dogs that consume bovine tissues, placentas, or uterine discharge are associated with a greater risk of transmitting the infection to cattle (Dijkstra *et al*., 2002), and have the potential of causing point source outbreaks of neosporosis (McAllister *et al*., 1996, 2000; Dijkstra *et al*., 2002).

Although *N. caninum* IFA serological cut-offs of 1:25 (Wouda *et al*., 1997a) or 1:80 (Barr *et al*., 1995) are considered to be specific for fetal sera, there is no well-established cut-off titer for newborn calves. Although precolostral titers in congenitally infected calves have been reported to be very high (≥ 1:1,280 or ≥1:5120) (Anderson *et al*., 1997; Conrad *et al*., 1993), the congenitally infected calf in the present report shows that a cut-off above 1:100 could give a false-negative result.

All hamsters in the present study showed no clinical illness, lacked lesions and no demonstrable parasites by HE and IHC (except hamster H3 of calf No. 2156 the brain of which showed multifocal non-suppurative areas by HE staining) but NC DNA was amplified from the brains of two hamsters (H2 and H3; inoculated with the brain of calf No.2156; see table 1). It is

possible that the number of parasites present in the calf brain homogenates might be too few to affect the hamsters. On the other hand, it is also possible that the parasites were not virulent as the hamsters were immunosuppressed with dexamethasone sodium phosphate. Because two of four hamsters were infected but with no clinical illness, it is also likely that hamsters were not susceptible enough to the parasites. Unlike the hamsters in this experiment, Djungarian hamsters (*Phodopus sungorus*), were very susceptible to NC (Uchida *et al*., 2003). They showed clinical signs and numerous tissue cysts in the brains 5 weeks post inoculation with NC tachyzoites. The possible reasons for the dissimilarities may be due to species difference, the virulence and the infective dose of the NC-parasite. Djungarian hamsters were challenged with 5×10^6 tachyzoites. Undoubtedly, compared to them, the brain homogenates in the present experiment could not contain that much. A similar pattern was reported in experiments with gerbils (*Meriones unguiculatus*) which were highly susceptible to NC (Dubey and Lindsay, 2000; Gondim *et al*., 2001). In contrast, Rodrigues *et al*. (2004) found no clinical signs and histological lesions in any of 18 gerbils infected with the brain homogenates of NC-seropositive buffaloes. Yet, NC-DNA was amplified from the brains of three gerbils. The authors suggested that NC isolates from their buffaloes were not virulent.

In conclusion, *N. caninum* antibody titers may drop or convert to seronegative status in chronically infected cows by the time of parturition, and this finding in 4 of 4 cows indicates that this could be a common occurrence. Similarly, the finding of an infected calf with a low antibody titer indicates that pre-colostral serology is not a fool-proof means of identifying calves with congenital *N. caninum* infections. These findings call into question conclusions of other studies that have estimated rates of endogenous transplacental transmission of this parasite based on serological tests at calving. Seronegativity of the cow and its calf at parturition while the calf was *Neospora*-positive by other means indicates the possibility of misinterpreting the serological results taken from cows and calves of similar situation. Serological cut-off values for both cows and calves should be set to a titer lower than the previous reports to avoid false negative. Serological tests should be done at different time points to correctly assess the seropositivity of the animal. It is still needed to further clarify whether the NC-isolation technique in this study should be used as a guideline or not. This is the first identification of tissue cyst in the brain of congenitally infected, normally born calf and confirmation of *Neospora* parasites by IHC, PCR and bioassay in Thailand.

CHAPTER III

THE IMPACT OF *Neospora caninum* ON THE REPRODUCTIVE PERFORMANCE OF COWS AND ESTIMATION OF ABORTION RISK FROM THE *Neospora* SEROLOGICAL STATUS OF COWS

3.1 Introduction

This chapter contains two studies. The first study was to investigate whether there were differences in the reproductive performance (number of AI per confirmed conception, age at first service, first service conception rate, age at first calving, calving to first insemination, calving to conception, calving intervals, placenta retention and stillbirth) between *Neospora* seropositive and seronegative cows. The aim of the other study was to estimate abortion risk from the *Neospora* serological status of cows.

3.2 The impact of *Neospora caninum* on the reproductive performance of cows

3.2.1 Materials and methods

3.2.1.1 *Study Herds and Design*

The study was conducted in the twelve *Neospora* seropositive dairy herds, having 4 to 29 Holstein Friesian (HF) crossbred cows (total = 216 cows). Seventy per cent of cows were ≥ 75% HF crossbreds and the others were between about 45 to 70%. These herds were located in Nakhon Pathom Province and they all were seropositive as determined by using competitive enzyme linked immunosorbent assay (cELISA) in 2001. These farms were established as early as 1978 and as late as 1997; 67 % (8/12) of them being established after 1991. They began with a few numbers of cows ranging from 2 to 7 animals. Poultry, ducks, swine and cats were also found on these farms. Nine farms had dogs ranging 1 to 4 in numbers. Dairy cows were reared in the confinements. All herds had a concrete floor and roofed with asbestos or corrugated iron-sheets. Generally the farm management practices were similar as they followed the guidance of Nongpho Dairy Cooperation. They used artificial insemination for the breeding of their animals in connection with the Artificial Insemination Center, Department of Livestock Development. Basically the animals were fed with corn- and grass-based roughages and concentrate sold by the Nongpho Dairy Cooperation. A longitudinal design was used for the comparison of reproductive performance between seropositive and seronegative cows in these farms.

3.2.1.2 *Blood Sample Collection and Serological Test*

Blood samples from all cows, including heifers over one year of age, were collected monthly for ten months starting from January to October 2004. The blood of dogs on the farms was also collected once during January sample collection. The sera were separated in the same collection day and stored at -20°C till tested. Antibodies against *N. caninum* in the sera of all dogs and cows were checked by using IFA (section 2.2.1.2). For *T. gondii* antibody test, dogs and only seropositive cows and aborting cows were checked by direct agglutination test as described in section 2.2.1.2.

3.2.1.3 *Data Collection*

The artificial insemination dates, number of services per confirmed conception of cows, and calving dates for individual cows were obtained from the Ratchaburi AI center and farm records covering over two years' period (2003-2004). Calving intervals and calving to conception dates were calculated from the records. As a nature, the data from the year 2002 was also included in the calculation of the calving intervals. Data collection for the study is summarized in Appendix F.

3.2.1.4 *Statistical Analysis*

The hypothesis of no differences in the reproductive performance of *Neospora* seropositive and seronegative cows was tested with analysis of variance by using General Linear Model (PROC GLM, SAS software). In the model, the effect of herd and serostatus of the cows were included as independent variables and reproductive performance parameters as dependent variables. For the analysis of first service conception rate, a Chi-square test was used and the risk of becoming nonpregnant in seropositive cows was estimated. Because some cows, including both seropositive and seronegative cows, were sold before the study was completed, their records were only partially included in the analysis.

3.2.2 Results

3.2.2.1 *Serology of Dogs and Cows*

The overall seroprevalence was 12.96% with a range of 4.76 to 30% among the herds (Table 3). At least one seropositive cow was detected from each farm. The antibody titers ranged from 1:200 to 1:3200 and 82% of the seropositive cows had a low titer of 1:200 (Table 4). Cows having the titers of ≥1:400 remained seropositive and nine lowly seropositive cows

(39.1%) became seronegative at the end of the study. No positive seroconversion in the seronegative cows was observed.

Among 16 dogs in nine farms, two were not catchable. Of 14 farm dogs tested with IFA, only one male dog, aged 2 years, from farm "A" was seropositive having a titer of 1:50. None of the cow and dog sera reacted with the *Toxoplasma* DAT test.

Table 3. Prevalence of antibodies to *N. caninum* in the dairy cows and the number of dogs on the 12 farms.

Farm	No. of Dogs	No. of cows	*N. caninum* serostatus of cows (IFA)		
			+ve	-ve	seroprevalence
A	3*	29	3	26	10.34%
B	1	18	3	15	16.66%
C	1	19	2	17	10.5%
D	No dogs	25	2	23	8%
E	No dogs	21	1	20	4.76%
F	No dogs	14	3	11	21.43%
G	3	4	1	3	25%
H	1	10	3	7	30%
I	3	15	3	12	20%
J	1	20	4	16	20%
K	1	24	2	22	8.33%
L	2	17	1	16	5.88%
Total	16	216	28	188	12.96%

* a dog, 2 yr old, was seropositive; 2 dogs uncatchable

Table 4. *Neospora* antibody titers and seroconversions of cows from the 12 dairy farms.

	No.	Titer* (Reciprocal) January 2004	%	Seroconversion October 2004	
				+VE to -VE	-VE to + VE
Seropositive animals	23	200	82.1	9	
	1	400	3.6	None	
	2	800	7.1	None	
	1	1600	3.6	None	
	1	3200	3.6	None	
Total	28		100		
Seronegative animals	188				None

***** Cut-off value for all tests from January to October: 1:200

3.2.2.2 *Reproductive Performance*

No significant differences (P>0.05) in all reproductive performance parameters between seropositive and seronegative cows were observed (Table 5). Seropositive cows, although not significantly different from the seronegative ones, tended to have a younger age at first service and at first calving, fewer numbers of AI per conception, shorter calving intervals and shorter calving to first service intervals. But with the age at first conception, seronegative cows tended to conceive at younger age. The significant differences of age at first service (P=.0005), the number of services per conception (P=0.0001) and calving to first service (P=0.0001) were found among herds.

A cross tabulation of NC serostatus of cows and the first service conception rate was shown in Table 6. The first service conception rate between seropositive and seronegative cows was also not significantly different (P>0.05). There was no risk of becoming not pregnant at the first service due to *Neospora* seropositivity (RR=0.818; 95%CI=0.602 to 1.163). Monthly first service conception rate had a similar picture (Table 7). Here also again, seropositive cows had a higher tendency of becoming pregnant than seronegative cows.

Table 5. Least square means (± SE) of different reproductive performance traits between seropositive and seronegative cows.

	Age at first service (d)	Age at first conception (d)	No. of AI per conception	Age at first calving (m)	Calving interval (d)	Calving to first Service (d)	Calving to conception (d)
Sero +ve cows	712.8±54.9 (n= 7)	714.3±89.1 (n=5)	2.7±0.41 (n=24)	28.7±3.42 (n=7)	443.3±37.1 (n=16)	102.1±10.8 (n=20)	217.9±32.7 (n=19)
Sero –ve cows	741.9±19.9 (n=56)	777.8±29.5 (n=50)	3.1±0.14 (n=166)	34.6±1.28 (n=63)	482.8±20.2 (n=78)	111.8±4.46 (n=135)	217.8±16.6 (n=95)
	P=0.615	P=0.50	P=0.375	P=0.1	P=0.319	P=0.4	P=0.99
Among herds	P=0.0005	P=0.160	P=0.0001	P=0.087	P=0.455	P=0.0001	P=0.63

Table 6. Cross-tabulation of serology of cows and first service conception rates.

	Nonpregnant	Pregnant	Total	Relative risk (95%CI)
Seropositive cows	14 (58.3%)	10 (41.7%)	24 100%	0.818 (0.602 – 1.163)
Seronegative cows	112 (71.3%)	45 (28.7%)	157 100%	
Total	126 (69.6%)	55 (30.4%)	181 100%	

CI = Confidence interval

Table 7. Cross tabulation of serological status of cows and conception rate by month.

Month	Serology	Pregnancy			RR (95% CI)
		No	Yes	Total	
January	Positive	6 75%	2 25%	8 100%	
	Negative	16 53.3%	14 46.7%	30 100%	
	total	22 57.9%	16 42.11%	38 100%	1.4 (0.84 – 2.37)
February	Positive	5 83.3%	1 16.7%	6 100%	
	Negative	20 80%	5 20%	25 100%	
	total	25 80.7%	6 19.3%	31 100%	1.04 (0.67 – 1.62)
March	Positive	1 33.3%	2 66.7%	3 100%	
	Negative	15 65.2%	8 34.8%	23 100%	
	total	16 61.5%	10 38.5%	26 100%	0.5 (0.1 -2.6)
April	Positive	0	1 100%	1 !00%	
	Negative	18 85.7%	3 14.3%	21 100%	
	total	18 81.8%	4 18.2%		0.29 (0.03 – 3.3)
May	Positive	3 100%	0	3 100%	
	Negative	27 87.1%	4 12.9%	31 100%	
	total	30 88.2%	4 11.8%	34 100%	1.15 (1.0 – 1.3)

Table 7. Continued

Month	Serology	Pregnancy			RR (95% CI)
		No	Yes	Total	
June	Positive	2 66.7%	1 33.3%	3	
	Negative	23 85.2%	4 14.8%	27 100%	
	total	25 83.3%	5 16.7%	30 100%	0.78 (0.35 – 1.77)
July	Positive	2 100%	0	2 100%	
	Negative	21 87.5%	3 12.5%	24 100%	
	total	23 88.5%	3 11.5	26 100%	1.14 (0.98 – 1.33)
August	Positive	1 100%	0	1 100%	
	Negative	16 69.6%	7 30.4%	23 100%	
	total	17 70.8%	7 29.2%	24 100%	1.44 (1.1 - 1.9)
September	Positive	0	0	0	
	Negative	12 84.6%	2 15.4%	14 100%	
	total	12 84.6%	2 15.4%	14 100%	Not determined
October	Positive	1 100%	0	1 100%	
	Negative	21 91.3%	2 8.7%	23 100%	
	total	22 91.7	2 8.3%	24 10-0%	1.1 (0.97 – 1.24)

3.2.2.3 *Retention of Placenta and Stillbirth*

The retention of placentas was observed in one seropositive aborting cow and in two seronegative aborting cows during the study period. Stillbirths were also not found.

3.2.3 Discussion

As expected the average seroprevalence of these farms was relatively high (12.96%) as compared to the previous report (5.46%; Kyaw et al., 2004). As no positive seroconversion in the seronegative cows was observed, there may be no new infections in these herds during the study period. The goals of all dairy farms are to achieve high reproductive efficiency of their cows. The goals set and the measures of the reproductive efficiency in dairy cows vary from farm to farm depending on the farm situation. Moreover, the performances are greatly influenced by many factors such as diseases, climates and nutrition (Farin and Slenning, 2001; Heinrichs and Radostits, 2001). *Neospora* has been one of alarming abortifacient diseases in cattle industry. The present study showed that seropositive cows and seronegative cows had not any significant differences in all reproductive performance parameters observed (Table 5) indicating no influential effect of *Neospora*-seropositivity on reproduction. This is in agreement with such traits as the number of first services and conception rates (Björkman *et al*., 1996; Jensen *et al*., 1999). Even in the *Neospora* outbreak farms, the subsequent effect, one season after the outbreak, on the risk of abortion, stillbirth and non-pregnancy was not significant (Waldner *et al*., 2001). In a Swedish study, the number of services required per confirmed conception was the same in both seropositive and seronegative cows (Björkman *et al*., 1996). But this was in contrast with the other Swedish report that showed higher number of services in seropositive cows than Swedish standard (2.2 vs. 1.7; Stenlund *et al*., 1999). Recently, it was reported that the fertility in the high producing cows were not affected by *Neospora* infection (López-Gatius et al., 2005). Also another recent study in Costa Rica showed no significant effects on calving interval and services per conception (Romero et al., 2005). The number of services per confirmed pregnancy in the present study (2.7 to 3.1) was higher than other Thai reports: 2.6 (Chantaraprateep and Humbert, 1994) and 2.8 (DLD, 2003). Although not significantly different in the performance in our cows, it is interesting that seropositive cows had a tendency of being better performance than seronegative ones. Also, despite indifference between seropositive and seronegative cows, there were significant differences (P=0.0005 to 0.0001) in the number of services per conception and calving to first service among herds (Table 5). This further pointed out that the reproductive performances of these cows were influenced by other factors than *Neospora* seropositivity. For the ease of comparison, the reproductive parameters in this study are described along with the standard values and reports of Thai studies (Table 8).

Table 8. Reproductive performances of the present study comparing with the other reports

Parameters	Present study Mean±SD	Reference			
		Adams *et al*., 1995 (Goals)*	Zwart and Jong, 1997**	Chantaraprateep and Humbert, 1994#	Pongpiachan *et al*., 2003#
Age at 1st service (d)	734±163	420-450	720	-	-
Age at 1st Conception (d)	773.7±161.4	-	765	-	-
No. of AI per confirmed pregnancy	3.1±2.1	1.9	-	2.6	2.3
Age at 1st calving (m)	34.4±8.9	24	34.7	29 to 33	-
Calving interval (d)	471±134	372 to 384	411 to 441	-	401
Calving to 1st service (d)	106.7±50.3	95 to 110	-	114	86.7 to 110
Calving to pregnancy (d)	213.5±131.3	-	-	186	139

*Reproductive performance goals
** Tropical standard
# Thai reports

In this study, the interval of calving to first service date is the only parameter which was similar to other report (Adams *et al*., 1995; Chantaraprateep and Humbert, 1994; Pongpiachan *et al.*, 2003). This period is a good indicator of efficiency of estrus detection (Adams *et al*., 1995). Estrus detection is one of important key factors for successful reproduction in dairy cattle. It seemed that this may not be a serious problem in these farms but the high number of services required for a confirmed pregnancy and long calving to conception intervals indicate infertility problems in these farms.

It was reported that most of the farms, even in well established and well organized farms, did not meet the standards (Farin and Slenning, 2001; Heinrichs and Radostits *et al*., 2001). But, obviously, all performance traits in this study, except calving to first service date, were considerably higher than the standards and other reports (Table 10). As discussed earlier, the infertility problem was not related to *N. caninum* infection. It should be noted that comparing the performances at different management systems and different climatic conditions may not be appropriate. For example the estrus period is generally shorter in the tropics because of poor nutrition and intensive suckling (Zwart and de Jong, 1997).

Hobson et al. (2004) reported that the placenta retention was associated with the *Neospora* abortions. It is difficult to compare as placenta retention in this study was not frequently found. Also stillbirth was not observed. Infertility was the main problem and this was reflected by the low reproductive performances in these farms regardless of seropositivity or seronegativity of the cows. Although culling was mainly due to infertility, it was found that some farms maintained cows even after 10 or more unsuccessful services.

In conclusion**,** the *Neospora* seropositivity of cows in the seropositive farms with low seroprevalence will not affect their reproductive performances.

3.3 Estimation of abortion risk from the *Neospora* serological status of cows

3.3.1 Materials and methods

3.3.1.1 *Study Herds and Design*

As in Section 3.2.1.1.

3.3.1.2 *Blood Sample Collection and Serological Test*

As in Section 3.2.1.2.

3.3.1.3 *Data Collection*

The abortion cases for individual cows were obtained from the Ratchaburi AI center and farm records. Data collection for the study is summarized in Appendix F.

3.3.1.4 *Statistical Analysis*

Chi-square test was used (1) to test if there was a difference in abortions between *Neospora* seropositive and seronegative cows, and (2) to estimate relative risk of abortions in seropositive cows. Because the frequency of abortions was very low and dispersed over the two years, incidence rate of abortions each month was not analysed. Nonpregnant cows due to infertility, cows with missing records and eligible heifers still not pregnant were not included in the analysis. The level of significant difference was determined at $p<0.05$.

3.3.2 Results

The frequency and distribution of abortions in the seropositive and seronegative cows from the 12 farms was shown in Table 9. Five of 12 farms (41.7%) had no abortion cases. Altogether, 11 abortion cases were observed. Three of 11 abortions (27.1%) were from the seropositive cows and occurred in the previous year 2003. The rest of the abortions were from the seronegative cows. Only 2 abortions were observed in 2004; they both were from the seronegative cows. Because of low frequency of abortions, a cross tabulation of serostatus of cows with the overall abortions was done and the results were shown in Table 10. The relative risk of seropositive cows being to be aborted was not significant (RR=2.25; CI= 0.64 to 7.9).

Table 9. Distribution of abortion cases in the 12 dairy farms.

Herd	Seroprevalence	No. Aborted		% aborted*
		+ cow	- cow	
A	10.3% (3/29)	0	2	7.4 (2/27)
B	16.7% (3/18)	0	0	0 (0/17)
C	10.5% (2/19)	0	1	6.3 (1/16)
D	8% (2/25)	0	0	0 (0/13)
E	4.8% (1/21)	1	0	8.3 (1/9)
F	21.4% (3/14)	0	0	0 (0/12)
G	25% (1/4)	0	0	0 (0/4)
H	30% (3/10)	1	0	10 (1/10)
I	20% (3/15)	0	2	15.4 (2/13)
J	20% (4/20)	1	1	11.1 (2/18)
K	8.33% (2/24)	0	2	9.5 (2/21)
L	5.88% (1/17)	0	0	0 (0/12)
Total	12.96% (28/216)	3	8	6.3 (11/175)

*nonpregnant, unrecorded or sold cows not included.

Table 10. Cross tabulation of cumulative abortions with the serostatus of cows.

	Abortion		Total	Relative Risk (95%CI)
	Aborted	Not aborted		
Seropositive	3 (12.0%)	22 (88.0%)	25 (100%)	
seronegative	8 (5.3%)	142 (94.7%)	150 (100%)	
Total	11 (6.3%)	164 (93.7%)	175 (100%)	2.25 (0.64 – 7.9)

3.3.3 Discussion

As discussed in the section 3.2.3, the *Neospora* seroprevalence of dairy cows of twelve seropositive farms in this study was relatively high (12.96%). Here, the abortion rate, 6.3% (Table 10), was well above the normal acceptable figure of 4% (Adams *et al*., 1995) indicating some reproductive problems. Moreover, the present analysis showed no difference in abortion risk (RR=2.25; CI= 0.64 to 7.9) between seropositive and seronegative cows. This is in contrast with the other reports where 2 to 3.5-fold increased risk of abortions in seropositive cows was found (Pare *et al*., 1997; Moen *et al*., 1998; Wouda *et al*., 1998 and Davison *et al*., 1999b). In one study, the abortion risk in seropositive cows was even 13-folds greater than seronegative ones (Hall *et al*., 2005). The reason may be that their study based on the abortion outbreaks caused by *N. caninum* while the present study was under sporadic abortions. The result is also not unexpected because all fetuses of *Neospora* infected cows may not be infected and all fetuses infected *in utero* may not be aborted; instead, they were even born normal and healthy (Paré *et al.*, 1996; 1997; Moen *et al.*, 1998). In this study, it was not possible to check the aborted fetuses for the *Neospora* infection. Therefore, their serostatus and histopathology were unknown. We have found in the previous experiment (chapter 2) that seropositive cows might become seronegative and the congenitally infected calf might also have a low antibody titer; also 3 of 4 calves born to the seropositive cows were not infected. These may cause more difficulty in the interpretation and in relating abortions to the *Neospora* infections. In the previous study there was there was no positive conversion in seronegative cows indicating no horizontal transmission during this study period.

In conclusion, our results indicate that under the condition of sporadic abortions in the *Neospora* seropositive herds, abortions due to *N. caninum* in cows may not be the problem, at least in our study area. The *Neospora* abortions were more pronounced during outbreaks by point-source infection (McAllister *et al*., 1996, 2000; Dijkstra *et al*., 2002) and subsequent years (Moen *et al*., 1998; Pfeiffer *et al*., 2002).

CHAPTER IV

GENERAL DISCUSSION AND CONCLUSIONS

As in the other countries Neosporosis, by seroprevalence studies with sera (Suteeraparp *et al*., 1999; Kyaw *et al*., 2004) and with bulk milks (Chanlun *et al*., 2002), is a common occurrence in dairy cattle in Thailand. The seroprevalence ranged from 0 to 70%. The report of significant *Neospora*-associated abortions (87.5%; 7 of 8 aborting cows were seropositive) by Suteeraparp *et al*. (1999) seemed to be a threatening signal for the dairy farmers but it was not confirmed by serology or pathology in the aborted fetuses. Five years later, attempt to identify the *Neospora* parasites in the aborted fetal tissues from the seropositive farms was made but the parasites were not labeled by IHC; only the placenta of one seropositive cow showed tachyzoites by IHC. Again, by serology, 41.7% (5/12) aborting cows were seropositive (Kyaw *et al*., 2003). In the present study, 12% of seropositive cows were aborted (Table 10). This is about 3 to 7 times less than the previous findings and there was no statistical association between abortion and seropositivity. But it was worth noting that 27.3% (3/11) of aborting cows were seropositive. On the other hand, the reproductive performance due to *Neospora* seropositivity was not significantly different; there was even a trend of better performance in the seropositive cows. But it is important to note that all the performance figures were much higher than the acceptable standards. More information is still needed to more accurately access the impact of *Neospora* on the dairy production.

For the first time in Thailand, the parasite was identified and confirmed in both aborted fetus and congenitally infected new born calf by serology, IHC, PCR and bioassay. One aborted fetal brain was labeled with IHC showing the parasites in the endothelial cells of the blood vessels and parenchyma of the brain. A tissue cyst was identified in the brain of a congenitally infected, healthy calf; NC DNA was also extracted and amplified by PCR from the brain of the same calf. Also the NC DNA was extracted and amplified from the brains of two hamsters inoculated with the brain homogenates of the infected calf. Isolation of the parasite by both direct culture and bioassay with hamster was not successful. NC DNA could not be extracted and amplified from the brains of seropositive cows and aborted IHC-positive fetus. Our finding supported the fact that detection of parasites in the fetal tissues is difficult even with PCR, the most sensitive method (De Marez *et al*., 1999; Rodrigues et al., 2004).

Generally dogs on the *Neospora* infected farms had a higher rate of antibody against *Neospora* (Sawada *et al*., 1998; Ould-Amrouche *et al.*, 1999; Mainar-Jaime *et al*., 1999; Wouda *et al*., 1999b; de Souza *et al*., 2002; Dijkstra *et al*., 2002 and Guimarães *et al*., 2004). But there

were also other reports that described the low rate of seropositive farm dogs. In a study in France, no seropositive dogs were present in 58% (7/12) of seropositive herds (Pitel *et al*., 2001). In this study, the number of seropositive dogs on the seropositive farms was low, (7.1%, 1/14) and it was consistent with the previous report, (1.22%, 1/82; Kyaw *et al*., 2004). It is possible that dogs in the present study were not infected with the parasite or they might not have detectable level of antibody. It is also worth noting that the dogs may not have serum antibodies even though they are shedding oocysts (McAllister *et al*., 1998a; Lindsay *et al*., 1999 and Dijkstra *et al*., 2001). This fact indicates the necessity for more understanding of the role of dogs in the *Neospora* epidemiology.

In conclusion:

1. The *N. caninum* parasites, both tachyzoites and tissue cyst, were identified from an aborted fetus and a congenitally infected new born calf.
2. *N. caninum* DNA was extracted, amplified and partially sequenced for 225 bp with 99% identities with the reference sequence NC1. The sequence was designated as Thai-B1 and deposited in the GenBank under accession number AY941177.
3. Further verification is needed to clarify whether Thai-B1 is avirulant strain and/or the golden hamsters are not susceptible enough so that it could be confirmed whether the isolation method in this study should be used as a guideline.
4. The abortion was not associated with the seropositivity of the cows under the condition of sporadic abortions with low prevalence rate.
5. The seropositive cows and their infected calves may have low antibody titers at the time of calving and vertical transmission rate of *Neospora* infected cows may be lower than the previous reports.
6. The reproductive performances were not different in both seropositive and seronegative cows under the present conditions but their performances were considerably lower than other reports indicating the influence of other factors than *N. caninum.*

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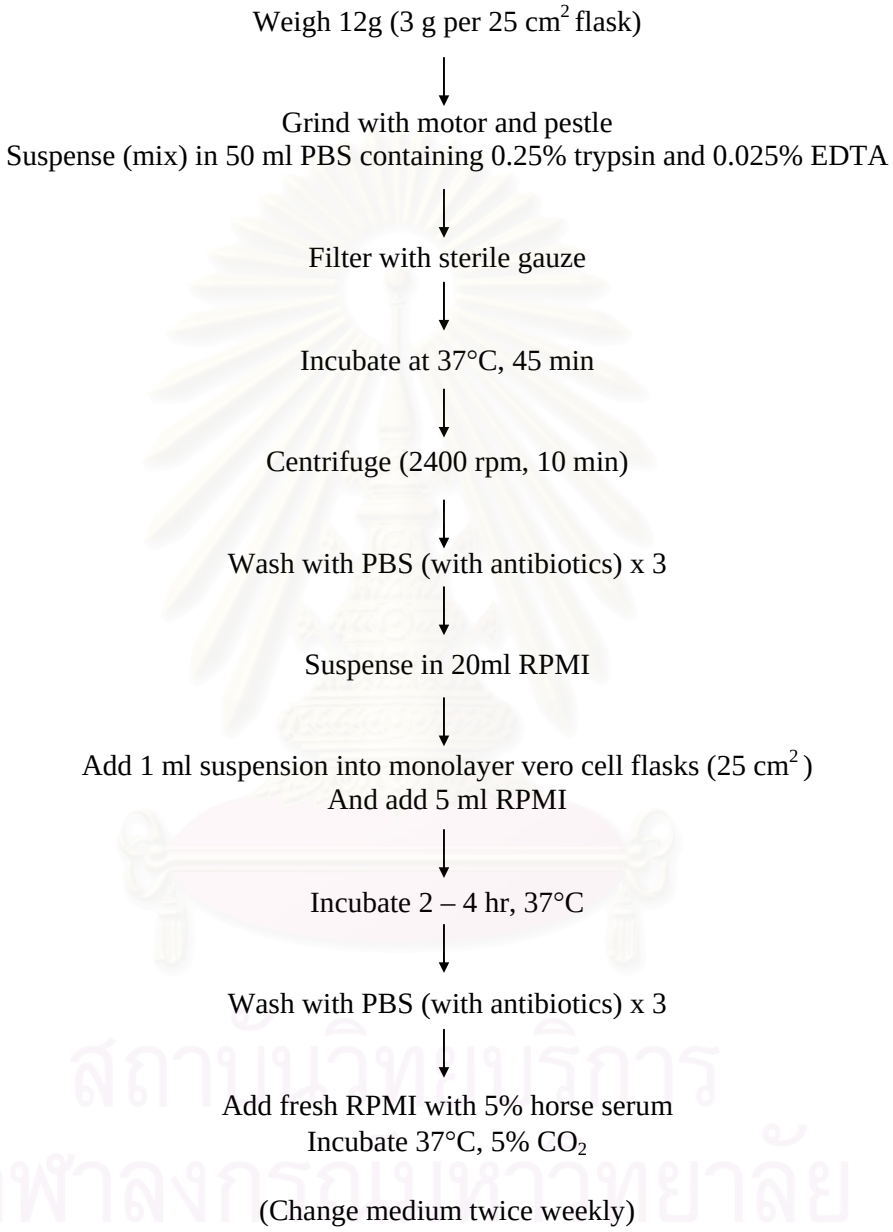
APPENDICES

Appendix A. Procedure to isolate *N. caninum* from brain tissues (Yamane et al., 1997)



Appendix B. Hematoxylin and Eosin (H&E) staining method of fetal tissues

Formalin-fixed, paraffin-embedded fetal tissues (cut at 4-5 μm thick sections)

↓

Deparaffinize in xylene (2 steps, 10 min each)

↓

Hydrated in the absolute alcohol (2 steps, 2 min each), 90% and 70% alcohol (2 min each)

↓

Washing the slides (5 min, in running water)

↓

Dipping in Mayer's Hematoxylin for 6 min and wash again

↓

Dipping in acid alcohol (10% glacial acetic acid in 95% alcohol)

↓

Washing in running water (5 min).

↓

Dipping (4 times) in saturated aqueous lithium carbonate (recolouring nuclei)

↓

Washing with running water (5 min).

↓

Counterstaining with eosin for 45 seconds.

↓

Dehydration by dipping the slides in 95% alcohol (3 dips), in absolute alcohol (2 steps, 2 min each), and in xylene (2 steps, 2 min each).

↓

Finally the slides were mounted with mounting media (DPX)

Appendix C. Immunohistochemical staining method

1. Cut 4-5 μm sections from formalin fixed, paraffin embedded samples
2. Deparaffinization
   Xylene (3 changes, 5 min each), Xylene+absoloute alcohol (2 min),
   Graduated alcohols (100%, 95%, 80%, 70%; 2 min each),
   Running water (5 min), DW (5 min), PBS (5 min)
3. Blocking endogenous peroxidase by 3% H_2O_2, 30 min, at room temperature
4. Wash with DW (5 min), PBS (2 x 5 min)
5. Antigen retrieval method
   Put slides on 0.01 M citrate buffer container.
   Heat in microwave (1200W, high power, 5 min)
6. Cool 30 min, wash with PBS (3 x 5 min)
7. Blocking non-specific binding antigen
   Apply 10% bovine serum albumin (BSA),
   Incubate at 37°C, in humid chamber, 30 min
8. Wash with PBS (3 x 5 min)
9. Apply primary antibody against *N. caninum* (1:10,000 dilution in PBS),
   Kept overnight at 4°C.
10. Wash with PBS (3 x 5 min)
11. Apply biotinylated secondary antibody (goat anti-rabbit IgG, 1:400 dilution in PBS),
    Incubate at 37°C, in humid chamber, 30 min
12. Wash with PBS (3 x 5 min)
13. Apply avidin-biotin complex (ABC) kit (A 4 μl +B 45 μl in PBS 5 ml; prepare 30 min before use); Incubate at 37°C, in humid chamber, 30 min
14. Wash with PBS (3 x 5 min)
15. Put slides in DAB substrate solution (0.075g DAB + Tris buffer 150 ml + 30% H_2O_2 50 μl), 8 min
16. Wash in running water, 5 min
17. Counterstain with hematoxylin, 30 sec
18. Wash in running water, 5 min
19. Dehydration
    95% ethanol, 100% ethanol (2 steps), xylene + absolute alcohol, xylene (2 steps): 2 min each
20. Mount slides with mounting media (DPX)

Appendix D. Gene extraction method from the brain tissue (QIAamp DNA mini kit)

25mg brain tissue (grind with liquid nitrogen in a mortar) + 180 µl buffer ATL
(In a 1.5 ml micro centrifuge tube)

↓

Add 20 µl proteinase K
Vortex, Incubate at 56°C (until complete lysis of tissue; 1-3 h)
(Vortex 3 times an hour); briefly centrifuge

↓

Briefly centrifuge to remove drops from the inside of the lid
Add 200 µl buffer AL
Vortex 15s, Incubate 70°C, 10 min
Briefly centrifuge

↓

Add 200 µl Ethanol (96-100%)
Vortex 15s, briefly centrifuge

↓

Apply the mixture to QIAamp spin column (in a 2 ml collection tube)
Without wetting the rim, close the cap
Centrifuge, 6000g (8000 rpm) 1 min,
Place QIAamp spin column in a clean 2 ml collection tube
(Discard the tube containing the filtrate)

↓

Open QIAamp spin column
Add 500 µl buffer AW1 without wetting the rim
Close cap, centrifuge 6000g (8000 rpm) 1 min
Place QIAamp spin column in a clean 2 ml collection tube
(Discard the collection tube containing the filtrate)

↓

Open QIAamp spin column
Add 500 µl buffer AW2 without wetting the rim
Close cap, centrifuge 20,000g (14, 000 rpm) 3 min

↓

Place QIAamp spin column in a clean 1.5 ml microcentrifuge tube
(Discard the collection tube containing the filtrate)
Open QIAamp spin column; Add 200 µl buffer AE
Incubate at room temperature 1 min
Centrifuge 6000g (8000 rpm) I min

Repeat last step
(DNA stored in buffer AE at –20°C for later use)
20 mg tissue – 10 to 30 µg of DNA in 400 µl

Appendix E. Aborted Feuses collected during 2002 to 2004 and their tissues.

Sr. No	Cow ID	Farm	Aborted date	Preg. (month)	Acces. No.	IFA (sera)		Tissues collected
						Dam	Fetus	
1	3131	B	4/8/03	8	3NP303	-ve	NA	Brain, heart, liver, kidney, placenta
2	44040	B	9/7/03	8	3NP304	-ve	-ve	Brain
3	071	B	4/8/03	5	3NP305	-ve	-ve	Brain, heart, lung, liver, kidney
4	18	B	29/8/03	7	3NP306	-ve	-ve	Brain, heart, liver, kidney, placenta
5	249	B	21/1/03	5.5	3NP307	-ve	-ve	Brain, heart, liver, kidney, placenta
6	097	N	2/7/03	3	3NP308	-ve	-ve	Brain, heart, liver, kidney
7	088	B	11/8/03	7	3NP309	-ve	-ve	Brain, heart, lung, liver, kidney
8	3144	N	10/7/03	7	3NP310	-ve	-ve	Brain, heart, lung, liver, kidney
9	472	B	8/12/03	4	2NP485	-ve	NA	Brain, heart, lung, liver, kidney
10	43074	B	20/11/03	6.5	2NP469	-ve	NA	Brain, heart
11	015	B	29/7/02	6	2NP314	-ve	NA	Brain
12	42096	N	26/7/02	5	2NP293	-ve	NA	Brain, heart, liver, kidney
13	3096	N	27/4/03	7.5	-	-ve	-ve	Brain, heart, lung, liver, , kidney
14	1010	B	18/12/03	7.5	3NP443	-ve	-ve	Brain, heart, lung, liver, , kidney
15	4130	N	24/12/03	6.5	3NP445	-ve	-ve	Brain, heart, lung,
16	4048	B	1/1/04	7.5	4NP27	-ve	-ve	Brain, heart, lung, liver, kidney, placenta
17	609	B	24/1/04	8.5	4NP60	-ve	-ve	Brain, heart, lung, liver, kidney
18	001	N	20/8/03	4	4NP61	-ve	-ve	Heart, liver, lung, placenta
19	**251***	B	9/3/04	5	4NP81	+ve (1:400)	NA	Brain, placenta
20	3055	B	24/12/03	9	3NP444	-ve	NA	Brain
21	3144	N	8/8/04	4	-	-ve	-ve	Brain, heart, lung, liver, kidney, placenta
22	180	B	24/12/03	5	-	-ve	NA	Brain

NA = Not available; B = Bangbung; N = Nongulam.
* Tachyzoites were observed in the brain sections by IHC.

Appendix F. Farm data collection

Farm ID…… Location …… Owner …….

Farm records

1. Year established ……. No of cows at the beginning ……

At present Number of cows ……

Number heifer ……..

Number of calves …….

2. Introduction of replacement heifer/cow (if any)

Year …… No. of animals …… Source ……. Cow ID ……

Year …… No. of animals …… Source ……. Cow ID ……

Year …… No. of animals …… Source ……. Cow ID ……

3. Presence of other animals dogs, Poultry, Duck, geese

Individual cow records

Cow ID …… Name …….…….. Birth date …….

1. Age at first service (month)
2. Age at first calving (months)
3. AI dates
4. Abortions (yes/no). If yes:

	AI date	Aborted date	Gestation period
1st abortion			
2nd abortion			
3rd abortion			

5. Retained placenta (yes/no)
6. Calving dates
7. Sex of calf (dead/alive)
8. Aborting cows - kept or culled …..…….

Culling reasons Mastitis, Parturient paresis, Lameness, Infertility etc.

Blood and data collection period: January to October 2004

BIOGRAPHY

Mr. Than Kyaw, a Myanmar student, was born in central Myanmar in 1951. He obtained Bachelor of Veterinary Science degree from the Institute of Veterinary Science, Yangon, in 1973. He worked as a demonstrator in the Department of Animal Science at the Institute of Agriculture from 1974 to 1986. He studied at Lincoln University, Christchurch, New Zealand, for his Diploma in Agricultural Science in 1982 and at Melbourne University, Australia, for his Master of Agricultural Studies emphasizing environmental effect on the performance of layers from 1987 to 1989. He then worked as assistant lecturer, lecturer and associate professor in the Department of Animal Husbandry at the University of Veterinary Science, Yezin, until 2000. In 2001, he was selected as a doctoral student by the Ministry of Livestock and Fisheries, Myanmar to study ruminant reproduction in the Department of Obstetrics, Gynaecology and Reproduction at the Chulalongkorn University with a financial support by Charoen Pokphand Foods Public Company Limited, Thailand. He obtained Master of Science degree in theriogenology from the Chulalongkorn University in 2003. His research area has been abortions in dairy cows related to the protozoan parasite *Neospora caninum*. The following papers have been published during his study.

1. Kyaw T, Virakul P, Muangyai M and Banlunara W. 2003. First identification of *Neospora caninum* in Thailand. Thai J. Vet. Med. Vol. 33 (4):97-102.
2. Kyaw T, Virakul P, Muangyai M and Suwimonteerabutr J. 2004. *Neospora caninum* seroprevalence in dairy cattle in central Thailand. Vet. Parasitol. 121:255–263
3. Kyaw T, Suwimonteerabutr J, Virakul P, Lohachit C and Kalpravidh W. 2005. Seronegative conversion in four *Neospora caninum*-infected cows, with a low rate of transplacental transmission. Vet. Parasitol. 131:145-150.